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# Carnitine and O-acylcarnitines in *Pseudomonas aeruginosa*: metabolism, transport, and regulation

Jamie Meadows  
*University of Vermont*

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CARNITINE AND *O*-ACYLCARNITINES IN *PSEUDOMONAS AERUINGOSA*:  
METABOLISM, TRANSPORT, AND REGULATION

A Dissertation Presented

by

Jamie A. Meadows

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy  
Specializing in Microbiology and Molecular Genetics

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Dissertation Examination Committee:

Matthew J. Wargo, Ph.D., Advisor  
Mary J. Dunlop, Ph.D., Chairperson  
Christopher D. Huston, M.D.  
Keith P. Mintz, Ph.D.  
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

## ABSTRACT

*Pseudomonas aeruginosa* is found in numerous environments and is an opportunistic pathogen affecting those who are immunocompromised. Its large genome encodes tremendous metabolic and regulatory diversity that enables *P. aeruginosa* to adapt to various environments. We are interested in how *P. aeruginosa* senses and responds to the host-derived compounds, carnitine and acylcarnitines. Acylcarnitines can be hydrolyzed to carnitine, where the liberated carnitine and its catabolic product glycine betaine can be used as osmoprotectants, for induction of the virulence factor phospholipase C, and as sole carbon, nitrogen, and energy sources. *P. aeruginosa* is incapable of *de novo* synthesis of carnitine and acylcarnitines and therefore imports these compounds from exogenous source. Short-chain acylcarnitines are imported by the ABC transporter CaiX-CbcWV. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically and the liberated carnitine is transported through CaiX-CbcWV. Once in the cytoplasm, short-chain acylcarnitines are hydrolyzed by the L-enantiomer specific hydrolase, HocS. The transcriptional regulator CdhR is divergently transcribed from the carnitine catabolism operon and we have identified the upstream activating region, the binding site sequence, and essential residues required for CdhR binding and induction of the carnitine operon. Carnitine catabolism is repressed by glucose and glycine betaine at the transcriptional level. Furthermore, using two different *cdhR* translational fusions we show that CdhR enhances its own expression and that GbdR, a related transcription factor, contributes to *cdhR* expression by enhancing the level of basal expression. These studies are the first to determine the mechanism of *O*-acylcarnitine transport, metabolism, and the regulation of these processes, which contribute to utilization of these compounds for *P. aeruginosa* survival in diverse environments.

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## **DEDICATION**

I would like to dedicate this dissertation to David V. Pollack, Ph.D. You are my inspiration and you gave me the courage to take on this endeavor, and for that I am forever grateful.

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## **CHAPTER 1:**

### **INTRODUCTION**

Jamie A. Meadows <sup>1</sup> and Matthew J. Wargo <sup>1,2</sup>

<sup>1</sup> Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, VT, USA

<sup>2</sup> The Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT, USA



## 1.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative bacterium that is ubiquitous in nature. *P. aeruginosa* is capable of living in many different environments due to its large genome that encodes diverse metabolic pathways. The genome is over 6.2 megabases and has over 5,570 predicted open reading frames that enable tremendous functional diversity [1]. With over 10 % of the genome predicted to be involved in metabolism and 8.4 % to regulation of gene expression, it's no wonder *P. aeruginosa* readily adapts to changing environmental conditions and nutrient sources [1]. Even though its respiration is primarily aerobic, *P. aeruginosa* can respire anaerobically using nitrate as an electron acceptor in the absence of oxygen. *P. aeruginosa* has a polar monoflagellum and Type IV motile pili making it highly motile, allowing the bacterium to actively find nutrients, attach to surfaces, and seek desirable environments [2]. Furthermore, it is this versatility that makes *P. aeruginosa* such a widespread pathogen [3], causing disease in plants [4, 5], nematodes [6], insects [7], and most notably, as an opportunistic pathogen in immunocompromised people.

To successfully cause infection, *P. aeruginosa* must incorporate and metabolize molecules derived from the host. General maintenance and expression of virulence factors are minimal requirements, while efficient catabolism and resultant growth can lead to successful colonization and improved evasion of immune-mediated clearance. The host-derived compound carnitine is abundant in host tissues and is readily used by *P. aeruginosa* [8]. Carnitine can be transported into the cell or acquired from metabolic

precursors, where it can serve directly as a compatible solute for stress protection, or be metabolized through one of a few distinct pathways as a nutrient source [9].

#### **1.1.1. Infections caused by *P. aeruginosa*: acute and chronic**

*P. aeruginosa* is one of the leading causes of nosocomial infections in the world [10] and often spreads from contaminated hospital materials (bedding, floors, sinks, etc.) and hospital personnel [11], although the actual routes of transmission from the contaminated sources to the patients have not been definitively shown. When *P. aeruginosa* establishes an infection it can develop into either an acute or chronic infection. This process is regulated via internal and external signals, often related to metabolism and nutrient acquisition, that alter motility and virulence expression to promote either an acute or chronic infection [12, 13].

Acute infections are severe, progressive infections that can result in septicemia and death if not treated properly. Virulence factors and toxins are often associated with acute infections. The exoenzyme type 3 secretion system (T3SS) of *P. aeruginosa* significantly enhances the pathogens ability to cause acute lung disease [14]. The Exotoxin (Exo) T and ExoS are GTPase-activating proteins that alter host actin cytoskeleton [15-17], while ExoU has patatin-like phospholipase activity affecting lipid metabolism and membrane integrity [18]. Together these exotoxins decrease host wound healing, cell motility and phagocytic capabilities, leading to septicemia and death [19]. People with severe burns are highly susceptible to infection due to breach in the natural skin barrier [11]. *P. aeruginosa* is the leading cause of infection in burns because

elements such as elastase, exotoxin A, phospholipases, quorum sensing, and the ability to be motile, enable *P. aeruginosa* to persist in the wound and disseminate [20, 21]. Other common acute infections are folliculitis [22], ulcerative keratitis [11], urinary tract infections [23], green nail syndrome [24], and episodes of pulmonary exacerbation in the lung diseases cystic fibrosis and chronic obstructive pulmonary disorder [25].

Chronic infections are those that can last months to a lifetime and are slow progressing. Cystic fibrosis (CF) is an autosomal recessive disorder caused from a mutation in the CF transmembrane conductance regulator (CFTR) gene, an ABC transporter that moves chloride ions across epithelial cell membranes. CF affects many organ systems but the altered lung physiology is currently the leading cause of death. The dysfunction of water balance across the respiratory epithelium and resultant impact on mucociliary clearance leads to a thick viscous mucus layer, a perfect environment for bacterial colonization [26]. After childhood, *P. aeruginosa* is the most common pathogen in the lungs and generally persists as a chronic infection despite extensive eradication regimes [27]. Infection of the lungs and the resultant lung function decline is the leading cause of mortality and morbidity in CF patients and more than 95% of CF patients die from respiratory failure, although only 70% of these are now from *P. aeruginosa* [28, 29]. In chronic infections, *P. aeruginosa* cells form biofilms, repress flagella expression, change their metabolic profile, and can become phenotypically mucoid by producing alginate, enhancing antibiotic resistance and impairing clearance by the host [25, 30].

### 1.1.2. Pathogenicity

The ability of *P. aeruginosa* to infect a wide range of hosts and cause significant disease and mortality is mediated by numerous factors that can be categorized as either virulence factors or virulence determinants [31]. Virulence factors, in the strictest definition, are secreted from the cell and damage the host. The most important group of secreted virulence factors are those secreted from the type three secretion systems (T3SS). The T3SS apparatus is a syringe-like complex that injects effector proteins directly into host cells to manipulate them [14]. In addition to T3SS, proteins can be secreted into the environment. Exotoxin A inhibits protein biosynthesis by inactivating elongation factor 2, resulting in cell death [32]. Phospholipase C, encoded by *plcH*, is secreted and cleaves phosphatidylcholine and sphingomyelin, components of eukaryotic cell membranes and lung surfactant. PlcH activity has been found to negatively impact lung function during infection, and can function to induce a proinflammatory response, suppress oxidative burst in neutrophils, degrade pulmonary surfactant, and increase endothelial cell death [33-38]. Elastases and proteases are other secreted virulence factors used by *P. aeruginosa* [11]. One of the most visually recognized virulence factors is pyocyanin because of its blue color. Pyocyanin is a redox-active secondary metabolite that not only kills eukaryotic host cells but also has the capacity to kill other bacteria through a myriad of mechanisms [31, 39].

Virulence determinants are factors that contribute to infection and disease, but not those that are for housekeeping maintenance [40]. Motility by flagella and pili are virulence determinants that contribute to pathogenicity through adherence, persistence in

wound sites, and dissemination throughout the host [41]. Quorum sensing is a virulence determinant used by bacteria to communicate and determine cell population density, and adjusts gene expression accordingly, impacting virulence [42, 43]. It was recently demonstrated that quorum sensing activates dispersal of biofilms [44], a community-based lifestyle. Biofilms are a developmental process leading to long duration attachment to a surface, where the cells are highly resistant to antimicrobials making it difficult to eradicate the bacteria [42]. A virulence determinant often overlooked is catabolism of host compounds. For a pathogen to colonize the host, the bacterium must acquire and catabolize host compounds to serve as nutrients, which are discussed in greater detail later in the chapter.

*P. aeruginosa* is a successful opportunistic pathogen because of metabolic diversity, secreted virulence factors, and resistance to antimicrobials and drug therapies. Virulence and antibiotic resistance are not mutually exclusive; alginate production, quorum sensing, and biofilm formation directly contribute to drug resistance. Resistance is largely attributed to intrinsic features in the microbe. For starters, the *P. aeruginosa* outer membrane is more restrictive than many other Gram-negative bacteria, with a permeability 12-100 times lower than in *E. coli* [45], excluding passage of many antibiotic compounds. Efflux pumps contribute to antibiotic resistance by pumping those antibiotics that passed the limited permeability barrier of the outer membrane back out of the cell. Overexpression of the MexAB-OprM, MexXY-OprM, and MexEF-OprN efflux systems removes  $\beta$ -lactams, quinolones, aminoglycosides, carbapenems, and disinfectants, respectively [46]. Another intrinsic resistance method that *P. aeruginosa*

possess is a chromosomal *ampC* gene, encoding a periplasmic  $\beta$ -lactamase that can inactivate  $\beta$ -lactam antibiotics [47]. Horizontal gene transfer and mutations can be acquired, providing additional mechanisms to escape the death blows from antibiotics and drugs.

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## **1.2 Carnitine in bacterial physiology and metabolism**

### **1.2.1. Summary**

Carnitine is a quaternary amine compound found at high concentration in animal tissues, particularly muscle, and is most well-studied for its contribution to fatty acid transport into mitochondria. In bacteria, carnitine is an important osmoprotectant and can also enhance thermotolerance, cryotolerance, and barotolerance. Carnitine can be transported into the cell or acquired from metabolic precursors, where it can serve directly as a compatible solute for stress protection, or be metabolized through one of a few distinct pathways as a nutrient source. In this review, we summarize what is known about carnitine physiology and metabolism in bacteria. In particular, recent advances in the aerobic and anaerobic metabolic pathways as well as the use of carnitine as an electron acceptor have addressed some long-standing questions in the field.

### **1.2.2. Introduction**

Carnitine (gamma-trimethylamino-beta-hydroxybutyric acid) (Fig. 1) is a quaternary amine compound that can be produced by all domains of life and was discovered in muscle extract in 1905 by Gulewitsch and Krimberg (Gulewitsch & Krimberg, 1905), and Kutscher (Kutscher, 1905). It was shown to be essential for larval development of the mealworm, *Tenebrio molitor*, and was originally designated vitamin B<sub>T</sub> based on this requirement. Later it was discovered that carnitine can be synthesized in mammals and is now considered to be a quasi-nutrient or conditionally essential nutrient (Flanagan *et al.*, 2010), as neonates have reduced biosynthesis and rely on placental

transfer of carnitine in utero and exogenous sources after birth (Combs Jr., 2012). Fifty years after the discovery of carnitine, it was demonstrated that assorted Gram positive and Gram negative bacteria could use carnitine in either aerobic or anaerobic environments for a variety of cellular functions, including as an electron acceptor, as a compatible solute to survive environmental insults, or as a sole carbon, nitrogen, and energy source. Bacterial carnitine metabolism was most recently reviewed fifteen years ago (Bieber, 1988; Bremer, 1983; Kleber, 1997; Rebouche & Seim, 1998), and the field has seen important advances. This review summarizes what we knew at the time of the previous reviews and emphasizes what we have learned since, including: (i) how anaerobic bacteria synthesize and utilize crotonobetaine and carnitine as final electron acceptors, (ii) the impact of carnitine degradation by the intestinal microbiota and the genes responsible for this anaerobic conversion, (iii) the genes involved in aerobic degradation of carnitine, and (iv) how carnitine as a compatible solute impacts survival within and outside of the host.

### **1.2.3. Carnitine in the environment**

Recent work makes it clear that while animals represent the most readily accessible source of carnitine, carnitine is often present and sometimes abundant in soil and natural waters. Quaternary ammonium compounds are abundant in a number of soil ecosystems, including comprising a quarter of the most abundant organic nitrogen compounds in the soil water of a sub-alpine grassland (Warren, 2013a). In this environment, carnitine was the most abundant quaternary ammonium compound (0.49

μM) and third most abundant soluble nitrogen compound overall, while acetylcarnitine was present at a slightly lower concentration (0.33 μM) (Warren, 2013a). It is apparent that carnitine concentration varies depending on sample location (Warren, 2013b), but there is need for a more thorough quantification of carnitine in other environments. The carnitine levels in soil and water may vary dependent on the bacterial flora at the site and whether the bacteria inhabiting those environments are capable of carnitine metabolism. The presence and utility of carnitine in the environment is supported, in part, by the number of bacteria capable of carnitine metabolism, including a few newly identified species such as *Burkholderia caribensis* (Achouak *et al.*, 1999), *Bacillus decisifrondis* (Zhang *et al.*, 2007), *Pseudomonas kilonensis* (Sikorski *et al.*, 2001) from soil, and *Shewanella pacifica* from sea water (Ivanova *et al.*, 2004).

#### **1.2.4. The importance of carnitine to animals (like us)**

Carnitine is most abundantly associated with animals, and its physiology in animals provides an important backdrop to our review of microbial processes. Carnitine is a zwitterion and can exist as either D- or L-enantiomers, but the D stereoisomer is not utilized for normal physiology in animals and can inhibit acylcarnitine transferases, thereby resulting in tissue depletion of L-carnitine (Bieber, 1988). Therefore, unless specifically noted, we are discussing L-carnitine. Animals use the L-carnitine shuttle to transport long to short chain fatty acids in and out of the mitochondria by reversibly esterifying the beta carbon hydroxyl group with a fatty acid to form *O*-acylcarnitine (Fig. 1). Beta oxidation of very long- chain fatty acids starts in the peroxisome, and once they

have been converted to medium- or short fatty acid chain lengths, carnitine is employed to traffic them out of the peroxisome and into the mitochondria where beta oxidation is completed (Reddy & Hashimoto, 2001; Steiber *et al.*, 2004; Wanders & Waterham, 2006). Acetyl-CoA derived from beta oxidation can be used to generate ATP via the TCA cycle, while beta oxidation allows the cell to maintain the acetyl-CoA:CoA ratio, and enables removal of specific harmful acylcarnitines derived from endogenous substances or from xenobiotics (Bieber, 1988). In humans, about 95% of total carnitine is found in skeletal and cardiac muscle, with the remaining 5% circulates in the plasma (Cave *et al.*, 2008). Approximately 75% of carnitine is obtained through diet; with foods from animal origin like meat and dairy having the highest carnitine content (Steiber *et al.*, 2004). The remaining 25% of carnitine is synthesized endogenously from the essential amino acids L-methionine and L-lysine in the liver, kidney, testes, and brain (Bremer, 1983; Flanagan *et al.*, 2010; Rebouche, 2014). The body maintains homeostatic levels of carnitine by balancing carnitine absorption from the small intestine lumen, reabsorption by the kidneys, and modest endogenous synthesis (Rebouche & Seim, 1998). Carnitine and acylcarnitines are primarily absorbed from the lumen of the small intestine where they are actively transported into enterocytes and diffuse past the serosal membrane into the circulatory system where they can then be transported into all other cells (Marciani *et al.*, 1991; Rebouche, 2004). Dietary carnitine that is not absorbed in the small intestines is metabolized by bacteria in the large intestine - there are no animal enzymes to break down carnitine (Rebouche & Chenard, 1991). Over the past decade, gut microbiome metabolism has become a topic receiving close review and recently Koeth and colleagues

associated the degradation of carnitine by intestinal microbiota with cardiovascular disease (CVD) and the promotion of atherosclerosis (Koeth *et al.*, 2013). Some intestinal bacteria can convert carnitine to trimethylamine, which is subsequently oxidized in the liver to the proatherogenic species trimethylamine-*N*-oxide (TMAO) (Koeth *et al.*, 2013), a metabolic pathway we discuss in more detail below. The role of bacterial metabolism of carnitine directly promoting CVD has been shown in multiple studies (Hartiala *et al.*, 2014; Koeth *et al.*, 2013; Kuka *et al.*, 2014), but is still up for discussion in the scientific and medical communities (Johri *et al.*, 2014). However, there is no debate that bacteria, intestinal and otherwise, utilize carnitine in many different ways for their benefit (Ussher *et al.*, 2013).

#### **1.2.5. The physiologic benefits of carnitine for bacteria**

Animals have been the focus of research on carnitine since its discovery in 1905 (Gulewitsch & Krimberg, 1905), and microbial carnitine metabolism wasn't described until almost fifty years later (Fraenkel & Friedman, 1957). In the intervening years, microbiologists have described the roles for carnitine in bacteria, where it can be used as a final electron acceptor, a compatible solute, or as a sole carbon, nitrogen, and energy source. Regardless of its eventual role, carnitine is transported into the bacterial cytosol by one of two different mechanisms. The first method is an ATP-dependent ABC transport system utilizing the canonical three subunits – a transmembrane domain, an ATPase, and a periplasmic binding protein (also known as a substrate binding protein). The second method of import is by BCCT (betaine-choline-carnitine) transporters that

can be driven by the sodium or proton motive force, but in many cases where carnitine is the substrate, often functions as a carnitine:gamma butyrobetaine antiporter (Ziegler *et al.*, 2010). Carnitine import or import of immediate precursors is critical for bacterial acquisition of carnitine, as Verheul *et al.* demonstrated that de novo synthesis of carnitine does not occur in *Escherichia coli* (Verheul *et al.*, 1998), and to the best of our knowledge, de novo synthesis of carnitine has not been demonstrated in any bacterial species.

#### Carnitine: an organic compatible solute

Outside of the cozy confines of the laboratory, bacteria are subject to constantly changing environments to which they must respond rapidly to survive and thrive. One way to accommodate stresses caused by changing water content, salt, temperature, or pressure is by synthesis or import of compatible solutes. Compatible solutes can be accumulated at high concentrations in the cytoplasm while not interfering with cellular processes, thus contribute to proper protein function and cellular homeostasis (Brown & Simpson, 1972). Compatible solutes are typically organized into categories: carbohydrates, amino acids, methylamines, methylsulfonium solutes, and specific inorganic ions. Organic solutes that are either non-charged or zwitterions with no net charge at physiological pH are preferred compatible solutes, the intracellular concentration of which are carefully regulated to maintain protein stability and cell physiology (Fitzsimmons *et al.*, 2012; Hoffmann *et al.*, 2013). Carnitine is an ideal compatible solute that can be imported and/or generated from direct precursors by many



bacteria and its abundance for infectious microbes in the host and presence in many natural environments suggests it is readily accessible (Warren, 2013a; Warren, 2013b).

### Osmoprotection

Drastic changes in water content in the environment can result in osmotic stress to the cell. Low external solute concentration is hypoosmotic, with resulting pressure to drive water into the cell causing an increase in cell turgor pressure. Conversely, increased external solute concentration due to added solutes or loss of water is hyperosmotic, with resulting pressure leading to water efflux from the cell, reducing turgor pressure. In addition to the altered physiology imparted by these turgor changes, both conditions can lead to cell death due to irreversible plasmolysis (loss of water) or cytolysis (too much water). To overcome osmotic stress, bacteria can either acquire or synthesize osmoprotectants, a process that is universal for bacteria. One way bacteria can overcome osmotic stress is by utilizing carnitine as an osmoprotectant and/or osmolyte.

### *Gram negative osmoprotection by carnitine*

The Gram negative opportunistic pathogen *Pseudomonas aeruginosa* uses carnitine as both an osmoprotectant and an osmolyte. Intracellular carnitine can be accumulated directly via transport from extracellular sources through a BCCT (Malek *et al.*, 2011) or ABC transporter (Chen *et al.*, 2010), or indirectly by degradation of *O*-acylcarnitines (Lucchesi *et al.*, 1995; Meadows & Wargo, 2013). *P. aeruginosa*, like many environmental proteobacteria, can also metabolize carnitine to the osmolyte glycine

betaine (Fig. 2) (Aurich *et al.*, 1967; Bastard *et al.*, 2014; Wargo & Hogan, 2009). *Escherichia coli* also use carnitine as an osmolyte, where it functions in aerobic, anaerobic, and high salt conditions (Verheul *et al.*, 1998). Import is primarily mediated through the ProU transport system, a substrate binding protein-dependent ABC transporter, along with modest uptake from the MFS-family transporter ProP in anaerobic and osmostressed aerobic cells. Although CaiT functions as a carnitine antiporter, its activity is not sufficient to confer osmoprotection (Verheul *et al.*, 1998). *Yersinia enterocolitica* also employs carnitine as an osmolyte, where unlike most Gram negative bacteria, nuclear magnetic resonance observations demonstrated that added carnitine was not metabolized to the more potent osmolyte glycine betaine (Park *et al.*, 1995).

#### *Gram positive osmoprotection by carnitine*

The lactic acid bacteria, *Tetragenococcus halophile* and *Lactobacillus plantarum*, can import and use D- and L-carnitine as osmolytes (Kets *et al.*, 1994; Robert *et al.*, 2000). *Brevibacterium linens* can also use D- and L-carnitine as osmolytes, and while L-carnitine can be metabolized to glycine betaine, it is further metabolized as a sole carbon or nitrogen source (Jebbar *et al.*, 1998). *Bacillus subtilis* imports D- and L-carnitine, acetylcarnitine, crotonobetaine, gamma-butyrobetaine, and octanoylcarnitine via the ABC transporter, OpuC. Both stereoisomers and the carnitine resulting from acetyl- and octanoylcarnitine degradation function as osmolytes to protect the cell from hyperosmotic stress (Kappes & Bremer, 1998). The carnitine generated or imported by *B. subtilis* cannot be metabolized further and therefore is thought to function primarily as an

osmoprotectant. *Staphylococcus aureus*, a common member of the skin and nasal flora that is an important opportunistic pathogen, uses carnitine as a compatible solute in high salt (Vilhelmsson & Miller, 2002).

Carnitine appears to be particularly important for the common foodborne pathogen *Listeria monocytogenes*. *L. monocytogenes* primarily imports carnitine through the OpuC ABC transporter (Fraser & O'Byrne, 2002; Fraser *et al.*, 2000; Verheul *et al.*, 1995; Verheul *et al.*, 1997), where it functions as an osmolyte in high salt conditions (Beumer *et al.*, 1994). Mice infected with *L. monocytogenes* perorally show that OpuC is important for survival and infection in the small intestine, and for the proliferation and dissemination of the bacteria into other organs, including the liver and spleen (Sleator *et al.*, 2001; Sleator *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2002). One of these studies postulated that bacterial destruction of the intestinal epithelial layer allows carnitine release, which then alleviates the effects of the hyperosmotic environment of the small intestine, where salt concentrations are two times higher than the blood (Sleator *et al.*, 2001). Additional mouse infections comparing mutants in the glycine betaine transport systems BetL and Gbu to mutants of OpuC led to the conclusion that carnitine transport is more important than glycine betaine for *Listeria* during infection (Sleator *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2002).

#### Beyond salt: the role of carnitine in cryoprotection, bile tolerance, and barotolerance

In addition to osmoprotection, compatible solutes can protect bacteria from additional sources of stress. *Listeria* and *Bacillus* have both been well-studied in relation

to carnitine uptake, and are good examples of bacteria that employ carnitine to protect against multiple stress conditions.

*Listeria* is a foodborne pathogen that has multiple lifestyles, thriving on decaying plant material and transiently living in the gastrointestinal tract of some animals, including humans. Any bacterium that lives or passes through the small intestine has to cope with bile salts, which aid in digestion of lipids, have antimicrobial activity, disrupt membranes and proteins, and induce oxidative stress and DNA damage (Begley *et al.*, 2005). The OpuC carnitine transport system in *L. monocytogenes* is important for protecting the bacteria against bile stress and its expression is coregulated with the bile efflux system Bile (Watson *et al.*, 2009). Mice infected perorally with an *opuC* mutant strain had significantly reduced numbers of bacteria in the feces and a decrease in systemic infection, measured as bacteria in the liver and spleen (Watson *et al.*, 2009). Carnitine enhances bile tolerance and is important for dissemination and survival in the small intestine (Gahan & Hill, 2014; Sleator & Hill, 2010; Watson *et al.*, 2009).

Unlike many bacteria, *L. monocytogenes* is capable of growing at refrigeration temperatures (4°C), making it a common contaminant of dairy, meats, fruits, and vegetables (Ryser & Marth, 2007). Cold or chill stress affects protein structure (Jaenicke, 1990), protein stability (Privalov & Gill, 1988), and membrane fluidity (Rudolph *et al.*, 1986), and compatible solutes, like carnitine, can alleviate these negative effects due to cold (Bayles & Wilkinson, 2000). The expression of the carnitine uptake system *opuC* is increased at low temperatures, allowing the cell to acquire carnitine and maintain growth

(Angelidis & Smith, 2003; Angelidis *et al.*, 2002; Sleator *et al.*, 2009; Wemekamp-Kamphuis *et al.*, 2004).

The ability to survive low temperature stress is not the sole domain of bacteria in our refrigerators, but rather a required feature of bacteria that inhabit the environment, where average soil surface temperatures range from 0 – 18 °C, average freshwater ranges from 3 – 25 °C, and while the average ocean surface temperature is ~17 °C the bulk of ocean water maintains a temperature of about 3 °C. Therefore, it is not surprising that successful environmental bacteria have also evolved pathways for cold tolerance that involve carnitine. In *Bacillus subtilis*, carnitine and its related metabolites crotonobetaine and gamma butyrobetaine have been shown to protect against both cold stress (15 °C) and heat stress (52 °C), via uptake through the OpuC transporter (Hoffmann and Bremer, 2011). It is likely that utilization of carnitine as a cryo- and thermo-protectant is not restricted to *Listeria* and *Bacillus* and the taxonomic breadth and ecological impact of this process requires further examination.

Pressure is another assault that *Listeria* has adapted to, as food processing uses high pressure for food preservation. *Listeria* exposed to elevated osmolarity and high pressure survives better and demonstrates substantial barotolerance when the compatible solute carnitine is present and imported into the cell (Smiddy *et al.*, 2004).

#### **1.2.6. Carnitine metabolism**

Carnitine can be utilized in different metabolic pathways to play a variety of physiologic roles. Figure 2 illustrates multiple described pathways for carnitine

metabolism and highlights bacteria in which particular reactions have been demonstrated. For each of the pathways described below, it is important to note that strain-specific utilization of carnitine via particular pathways can be governed by alternative regulation or the presence/absence of metabolic genes. For example, *Pseudomonas syringae* B728a carries the genes for carnitine conversion to glycine betaine, while *P. syringae* DC3000 has lost the entire carnitine catabolic operon (Chen & Beattie, 2007; Chen *et al.*, 2010).

#### Carnitine as a nutrient

Carnitine can be catabolized for use as a carbon source via two routes. The first pathway cleaves the backbone carbon-nitrogen bond to yield trimethylamine and malic semialdehyde, in which the latter enters the TCA cycle. The second route begins with the metabolism of carnitine to glycine betaine, which is then subjected to three successive demethylations to yield glycine, which can enter central metabolism. Carnitine can also be used as a sole nitrogen source, most commonly via the glycine betaine route, where glycine conversion to serine is followed by deamination to form pyruvate and ammonia. Formation of trimethylamine from carnitine leaves the nitrogen unusable for most of the organisms covered in this review, but many bacteria can use trimethylamine as a nitrogen source, carbon source, osmoprotectant, and, when oxidized to TMAO, functions as an electron acceptor (as reviewed in (Barrett & Kwan, 1985; Strom *et al.*, 1979; Yancey, 2005)).

### Carnitine to glycine betaine: a gateway reaction

Carnitine can be metabolized to glycine betaine via a multistep process, the first steps of which are encoded on genes located in the carnitine catabolism operon (Uanschou *et al.*, 2005; Wargo & Hogan, 2009). First, carnitine is converted to 3-dehydrocarnitine by the enzyme carnitine dehydrogenase (CDH, EC 1.1.1.108), an NAD<sup>+</sup>-dependent oxidoreductase. *P. aeruginosa* (Aurich *et al.*, 1967; Kleber & Aurich, 1967; Kleber *et al.*, 1967), *Xanthomonas translucens* (Arima *et al.*, 2010; Mori *et al.*, 1988), *Enterobacter* sp. (Hwang & Bang, 1997), *P. putida* (Kleber *et al.*, 1978), *P. fluorescens* (Hung & Kleber, 1985), *Burkholderia cepacia* (Dalmastri *et al.*, 2003), *Rhizobium* sp. (Arima *et al.*, 2010), and *Agrobacterium* sp. (Hanschmann *et al.*, 1996) all encode CDH enzymes that are specific for L-carnitine. 3-dehydrocarnitine is relatively unstable, therefore if there is no ATP or Coenzyme A (CoA) present, it will spontaneously decarboxylate to yield trimethylaminoacetone (N,N,N-trimethylaminopropanone) and carbon dioxide (Lindstedt *et al.*, 1967). If sufficient ATP and CoA are present, then 3-dehydrocarnitine is converted to acetylaceto-CoA and glycine betaine-CoA by the beta-keto acid cleavage enzyme (BKACE). The gene encoding the carnitine specific BKACE is located directly upstream of the CDH gene(s) in the carnitine catabolism operon (Uanschou *et al.*, 2005), and is designated *cdhC* in *P. aeruginosa* (Bastard *et al.*, 2014; Wargo & Hogan, 2009). The identification of this gene was part of a massive biochemical screen of various bacterial BKACE members, and proved very valuable for the identification of these CoA-dependent cleavage enzyme substrates (Bastard *et al.*, 2014). The glycine betaine-CoA derived from BKACE activity

is then converted to glycine betaine and CoA by a CoA transferase, likely the DhcAB enzyme in *P. aeruginosa* (Wargo & Hogan, 2009), which appears to function as a general amino acid CoA transferase (Palmer *et al.*, 2013). After carnitine is metabolized to glycine betaine, it can function as an osmolyte (as described above), or be utilized as a sole carbon, nitrogen, and energy source if the organism encodes the necessary enzymes, as in the case for *P. aeruginosa* (Wargo *et al.*, 2008), *Xanthamonas translucens* (Arima *et al.*, 2010; Mori *et al.*, 1988), *Enterobacter* sp. (Hwang & Bang, 1997), *Rhizobium* sp. (Arima *et al.*, 2010; Goldmann *et al.*, 1991), *Sinorhizobium meliloti* (Goldmann *et al.*, 1991), *Burkholderia cepacia* (Dalmastri *et al.*, 2003), and *Agrobacterium* sp. (Hanschmann *et al.*, 1996; Nobile & Deshusses, 1986), and metabolism and homeostasis of glycine betaine has recently been reviewed (Wargo, 2013).

#### Cleaving the backbone carbon-nitrogen bond

An alternative pathway to use carnitine as a sole carbon source is to cleave the carbon-nitrogen bond of carnitine to form trimethylamine and malic acid. It has been known since the mid 1960's that *Serratia marcescens* aerobically splits the carbon nitrogen bond of both D- and L-carnitine to form trimethylamine and malic acid, and that this reaction does not occur in anaerobic conditions since it requires uptake of oxygen (Unemoto *et al.*, 1966). Through the same reaction, *Acinetobacter calcoaceticus* can also use both D- and L-carnitine as sole carbon sources (Kleber, 1977; Miura-Fraboian *et al.*, 1982), yet the responsible enzymes remained elusive until recently. The genes encoding the enzymes that are essential for the conversion of carnitine to trimethylamine and malic



semialdehyde were recently identified as a two-subunit oxidoreductase in *Acinetobacter baumannii* (Zhu *et al.*, 2014), with CntA being a Rieske-family iron-sulfur cluster oxygenase, and CntB being the reductase. The CntAB proteins likely function analogously to GbcAB, which comprise the oxidoreductase that demethylates glycine betaine to dimethylglycine (Wargo *et al.*, 2008). Both sets of proteins cleave one of the C-N bonds in a quaternary amine compound, and these enzymes may represent a general evolutionary strategy for quaternary amine metabolism. Orthologs and homologs of the *cntAB* genes are found in a variety of gut microbiota: Gammaproteobacteria (*Klebsiella pneumoniae*, *E. coli*, *Citrobacter*, *Providencia*, and *Shigella*), Betaproteobacteria (*Achromobacter*), and Firmicutes (*Sporosarcina*) (Zhu *et al.*, 2014). The malic semialdehyde produced during this reaction is converted to malic acid, which enters the TCA cycle. Trimethylamine formed from carnitine by gut bacteria is correlated to human cardiovascular health (Koeth *et al.*, 2013), where it is oxidized to trimethylamine-*N*-oxide (TMAO) by hepatic flavin monooxygenases in the liver (Bennett *et al.*, 2013). Therefore, the identification of CntAB provides a target for monitoring gut microbiota capacity for trimethylamine production and is a critical step forward in our understanding of carnitine metabolism by bacteria.

#### Carnitine as a final electron acceptor

Bacteria that live strictly or transiently in anaerobic environments, where oxygen cannot serve as the final electron acceptor, can use alternate electron acceptors, including sulfates, nitrates, ferric iron, carbon dioxide, and fumarate, among others. In cases where

the common electron acceptors are not present, some Enterobacteriaceae (*E. coli*, *S. typhimurium*, and *Proteus* spp.) can use carnitine and its catabolic product crotonobetaine as final electron acceptors in the absence of oxygen and in the presence of additional carbon and nitrogen sources (Seim *et al.*, 1982a; Seim *et al.*, 1982b; Seim *et al.*, 1982c). Use of these compounds as electron acceptors is regulated by the transcriptional activator, CaiF, and the regulatory proteins CRP and FNR, which regulate expression of the divergently transcribed operons *caiTABCDE* and *fixABCX*; both required for carnitine metabolism in *E. coli* (Buchet *et al.*, 1998; Buchet *et al.*, 1999; Eichler *et al.*, 1995; Eichler *et al.*, 1996). For its role as an electron acceptor, carnitine is imported by the substrate:product antiporter, CaiT, which exchanges carnitine for its metabolic product gamma-butyrobetaine (Jung *et al.*, 1990; Jung *et al.*, 2002; Kalayil *et al.*, 2013). Co-expression of these two operons is induced by carnitine and crotonobetaine, but repressed by oxygen, glucose, gamma-butyrobetaine, and more desirable final electron acceptors such as nitrate and fumarate (Jung *et al.*, 1987; Seim *et al.*, 1982a; Seim *et al.*, 1982b). Expression of the carnitine metabolism genes in *E. coli* and *Proteus* spp. is also detectable in aerobic environments in the presence of inducing compounds, but to a much lesser extent than under anaerobic conditions (Elssner *et al.*, 1999; Engemann & Kleber, 2001; Obon *et al.*, 1999).

The *fix* operon is predicted to be involved in transferring electrons to carnitine (Eichler *et al.*, 1995; Eichler *et al.*, 1996; Walt & Kahn, 2002). The transformation of carnitine to crotonobetaine is reversible (Jung *et al.*, 1989) and was originally thought to occur via the single carnitine dehydratase, CaiB, with the cosubstrates gamma-

butyrobetainyl-CoA or crotonobetainyl-CoA (Eichler *et al.*, 1994a; Elssner *et al.*, 2000; Jung *et al.*, 1989). However Elssner *et al.* demonstrated that “L-carnitine dehydratase does not exist” and two enzymes are responsible for the reversible conversion of carnitine to crotonobetaine (Elssner *et al.*, 2001). CaiB is a CoA transferase that transfers CoA from gamma-butyrobetainyl-CoA, crotonobetainyl-CoA, or carnitiny-CoA to form gamma-butyrobetaine, crotonobetaine or carnitine. CaiD has enoyl-CoA hydratase activity and can dehydrate carnitiny-CoA to crotonobetainyl-CoA, or can hydrate crotonobetainyl-CoA to carnitiny-CoA, thus CaiD has two potential names: crotonobetainyl-CoA hydratase or carnitiny-CoA dehydratase. These enzymatic processes also allow carnitine to be synthesized from crotonobetaine and vice versa, (Figure 3) (Elssner *et al.*, 2001; Engemann *et al.*, 2001; Engemann *et al.*, 2005).

Crotonobetaine can be reduced to gamma-butyrobetaine by two proteins, CaiB, with one of the cosubstrates gamma-butyrobetainyl-CoA or crotonobetainyl-CoA, and the unidirectional enzyme crotonobetaine reductase, CaiA, which reduces crotonobetainyl-CoA to gamma-butyrobetainyl-CoA (Engemann *et al.*, 2005; Preusser *et al.*, 1999; Roth *et al.*, 1994). Two other proteins necessary for anaerobic carnitine catabolism are CaiC and CaiE. CaiC is a betaine:CoA ligase with CoA transferase activity *in vitro*, but is not sufficient *in vivo* to compensate for a *caiB* deletion. Therefore, CaiC is likely required for activation of trimethylammonium compounds (Bernal *et al.*, 2008; Eichler *et al.*, 1994b). The function of CaiE is still somewhat mysterious, but it is postulated to be required for activation or synthesis for an unknown cofactor necessary for carnitine metabolism (Eichler *et al.*, 1994b).

The enzymes for converting gamma-butyrobetaine into carnitine, best described in the Enterobacteriaceae, are predicted to be present in other bacterial groups. The Betaproteobacterium *Achromobacter cycloclast* and the Gammaproteobacterium *Acinetobacter calcoaceticus* are predicted to have similar enzymes as no gamma-butyrobetaine hydroxylase was detected during degradation of gamma butyrobetaine to carnitine. For *A. calcoaceticus*, after gamma butyrobetaine is degraded to carnitine, the carnitine can then be broken down to trimethylamine and malic acid where it uses it as a carbon source (Miura-Fraboïn *et al.*, 1982), whereas the fate of the newly synthesized carnitine was not investigated in *A. cycloclast* (Naidu *et al.*, 2001).

#### **1.2.7. Sensing and binding carnitine**

For osmotic adaptation to, or metabolism of carnitine, it is important for bacteria to regulate the expression of genes involved in these processes. To our knowledge, carnitine is sensed to regulate activity at the transcriptional level using so-called ‘one-component’ regulators, i.e. single polypeptides that sense ligand and regulate transcription. However, compared to our understanding of carnitine transport and metabolism, the detection of carnitine for transcriptional regulation is much more poorly understood. We know of only two transcription factors that sense carnitine to regulate transcription: CaiF in *E. coli* and CdhR in *Pseudomonas aeruginosa*. Both are transcriptional activators and induce transcription in response to carnitine (Buchet *et al.*, 1999; Wargo & Hogan, 2009), although importantly, direct interaction of either protein with carnitine has not been demonstrated.

In *E. coli*, the *cai* and *fix* operons (described above) are regulated by the global regulator CRP and the carnitine-responsive activator CaiF. The CaiF protein is small and is predicted to contain two AraC-like helix-turn-helix (HTH) domains and likely functions similar to the MarR ‘HTH-only’ activators (Buchet *et al.*, 1999). Interestingly, the lack of a canonical ligand-binding domain suggests that detection of the ligand occurs within what is typically thought of as the DNA binding domain of the protein or is mediated by an independently encoded sensor protein. Using the Phyre2 protein prediction server (Kelley & Sternberg, 2009), CaiF has high structural homology to GrlA from Enterohemorrhagic *E. coli* (EHEC), where the two-component response regulator GrlR has been shown to interact with GrlA, modulating transcriptional regulation (Creasey *et al.*, 2003; Russell *et al.*, 2007). While we have found no obvious GrlR homologs in the *fix* and *cai* operons, it remains a possibility that carnitine detection occurs via a GrlR ortholog or a cognate sensor kinase, like GlrK (Yamamoto *et al.*, 2005).

In *P. aeruginosa*, the transcription factor CdhR functions as the carnitine-responsive activator of the genes encoding the carnitine dehydrogenase enzymatic pathway (Wargo & Hogan, 2009). CdhR is a member of the glutamine amidotransferase-1 (GATase-1) sub-family of the AraC transcription factor family and has the canonical AraC-family structure consisting of an N-terminal ligand sensing and dimerization domain and a C-terminal DNA binding domain comprised of two HTH motifs. Orthologs of CdhR are found divergently transcribed from the aerobic carnitine dehydrogenase genes in a variety of organisms including many species in the families

Pseudomonadaceae, Burkholderiaceae, and Rhizobiaceae, as well as in *Mesorhizobium loti*, and *Silicibacter* sp. (Uanschou *et al.*, 2005). Given the conservation of the gene arrangement and predicted functions, we propose that further understanding of CdhR will yield insights into this regulation beyond *Pseudomonas*.

In Gram positive bacteria, the carnitine dehydrogenase operons contain a TetR-family transcription regulator that likely functions as the carnitine sensor for regulated expression of these operons. Compared to the Gram negative bacteria, presence of the carnitine dehydrogenase and its cognate TetR-like regulator is much more restricted, occurring in *Brevibacterium linens*, *Staphylococcus epidermidis*, *Streptomyces coelicolor*, and *Oceanobacillus* sp. (Uanschou *et al.*, 2005). For the first two organisms on this list, acquisition of this predicted horizontally-acquired operon makes teleological sense given the prevalence of carnitine in milk and in the skin, respectively.

An integral part of transport, metabolism, and transcriptional activation is binding of the target molecule. In the case of carnitine, crystal structures have been solved in complex that include carnitine in complex with the periplasmic binding protein OpuCC (Du *et al.*, 2011), the carnitine:gamma butyrobetaine antiporter CaiT (Schulze *et al.*, 2010; Tang *et al.*, 2010), and similar aromatic cage architecture of the quaternary amine binding site with gamma butyrobetaine has been determined for gamma butyrobetaine hydroxylase (Tars *et al.*, 2014). Therefore, like the situation for glycine betaine (Schiefner *et al.*, 2004) high affinity binding of carnitine and its metabolites seems to occur via an aromatic cage enabling the cation-pi interaction with the trimethylamine moiety coupled with properly spaced hydrogen bonding residues to

coordinate the carboxylic acid. While this binding arrangement has been demonstrated for transport and metabolic proteins, neither carnitine nor the related compounds choline or glycine betaine have been crystalized with their cognate transcriptional regulators. Given the specificity and affinity provided by the cation- $\pi$  interaction, it has been hypothesized that glycine betaine and carnitine-sensitive transcription factors utilize a functionally similar binding site (Bremer, 2011).

### **1.2.8. Multiple paths for carnitine acquisition**

#### De-novo carnitine synthesis

While bacteria can synthesize the quaternary amine compounds choline and glycine betaine from single unrelated carbon sources during growth on minimal media, such de novo synthesis of carnitine has not been demonstrated. Rather, as discussed below, carnitine is typically generated by metabolizing appropriate trimethylated precursors. While evidence for true de novo synthesis is lacking, N <sup>$\epsilon$</sup> -trimethyllysine is the starting point for mammalian carnitine synthesis (Vaz & Wanders, 2002), and bacteria are known to synthesize N <sup>$\epsilon$</sup> -trimethyllysine (Barbier *et al.*, 2013; Klagsbrun & Furano, 1975). Therefore, it remains a formal possibility that bacteria can use this N <sup>$\epsilon$</sup> -trimethyllysine to synthesize carnitine in a manner similar to mammals.

#### D-carnitine... not a dead end after all

Animals only synthesize and respond physiologically to L-carnitine, thus the presence of the D stereoisomer arises from bacterial processes on carnitine and its

derivatives, or from ingested food based on abiotic racemization. Despite the relative abundance of L-carnitine, some bacteria are capable of utilizing D-carnitine as a sole carbon and nitrogen source, including *Agrobacterium* sp., *Agrobacterium radiobacter*, and *Enterobacter* (Hanschmann & Kleber, 1997; Hwang & Bang, 1997; Kluttermann *et al.*, 2002). *Agrobacterium* expresses both an L-CDH and a D-CDH and its utilization of D-carnitine is dependent on the loss of chirality upon conversion of D-carnitine to the achiral 3-dehydrocarnitine (Hanschmann & Kleber, 1997). *Enterobacter* sp. KC-006 is also able to use D-carnitine as a sole carbon and nitrogen source. Mutations that significantly impair L-CDH activity permitted growth on D-carnitine as well as wild type, suggesting that a carnitine racemase was likely not responsible (Hwang, 1997). However, the D-carnitine metabolic pathway in *Enterobacter* has not been fully described. While *Enterobacter* likely does not have a carnitine racemase, there are bacteria that employ such an enzyme for D-carnitine metabolism. In *Pseudomonas* sp. AK1, a cytoplasmic carnitine racemase converts D-carnitine to L-carnitine, which is subsequently metabolized to glycine via glycine betaine to supply the cell with carbon and nitrogen (Monnich *et al.*, 1995). *E. coli* 044 K74 also expresses carnitine racemase activity, which is induced in the presence of L-carnitine or crotonobetaine and repressed by glucose, oxygen, and fumarate (Canovas *et al.*, 2003; Castellar *et al.*, 1998; Jung & Kleber, 1991). CaiD was initially suggested to function as the racemase, as the *caiD* gene is required for racemase activity (Eichler *et al.*, 1994b; Jung & Kleber, 1991; Jung *et al.*, 1987), however this has not been directly tested. CaiD is still postulated to be involved in racemization of D-carnitine, since CaiC was shown to activate D-carnitine by adding a



CoA group to produce D-carnitiny-CoA, which is then theorized to be converted to L-carnitiny-CoA by CaiD (Bernal *et al.*, 2008).

#### Carnitine synthesis by gamma-butyrobetaine hydroxylase

The direct route to the formation of carnitine from gamma-butyrobetaine occurs through the enzyme gamma-butyrobetaine hydroxylase (EC 1.14.11.1). It has been identified in *Pseudomonas* sp. AK1 (Lindstedt *et al.*, 1970a; Lindstedt *et al.*, 1970b; Ruetschi *et al.*, 1993), and *Pseudomonas* sp. L1 (Lu *et al.*, 2012), and both enzymes are homologous to the animal gamma-butyrobetaine hydroxylase in their requirement for oxygen and the cofactors iron, ascorbate, and  $\alpha$ -ketoglutarate (Lindstedt & Lindstedt, 1970; Lindstedt *et al.*, 1968).

#### Acylcarnitine

A fatty acid moiety can be conjugated to the third carbon of carnitine resulting in *O*-acylcarnitines (Fig. 1), which can serve as sources of carnitine but also can alter bacterial physiology directly (Nguyen *et al.*, 2012). *P. aeruginosa* can utilize acylcarnitines with 2-16 fatty acid chain lengths as sole carbon, nitrogen and energy sources, with the exception of octanoylcarnitine, although the reason for this utilization gap is unknown (Meadows & Wargo, 2013). Short-chain acylcarnitines (acetyl- and butyrylcarnitine) are hydrolyzed to L-carnitine and a short-chain fatty acid by the esterase HocS (Meadows & Wargo, 2013), while the medium- and long-chain acylcarnitine hydrolase(s) has not yet been identified. *P. putida* can utilize L-acylcarnitines with 10-16

fatty acid chain lengths as sole carbon and nitrogen sources (Kleber *et al.*, 1978). The L-enantiomers of short-chain acylcarnitines acetyl-, propionyl-, butyryl-, and iso-butyrylcarnitine, the medium chain lauroylcarnitine, and the long-chain palmitoylcarnitine can be hydrolyzed to carnitine in *Acinetobacter calcoaceticus* (Kleber *et al.*, 1977). Hydrolysis of D- and L-octanoylcarnitine has been assessed for a consortium of yeast, bacteria, and fungi resulting in the findings that *B. subtilis* ATCC 6633, *B. subtilis* sp. IMAM, and *Penicillium notatum* IMAM were capable of hydrolyzing D- and L-octanoylcarnitine, while *P. fluorescens* IMAM, *Rhodotorula gracilis* IMAM, and *Fusarium oxysporum* sp. *lini* IMAM had specificity for the L-enantiomer only. However, in this study, the biological function of the resulting carnitine or fatty acid in these strains was not assessed (Aragozzini *et al.*, 1986). An acylcarnitine hydrolase has also been purified from an *Alcaligenes* species and was capable of hydrolyzing acetyl-, propionyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoylcarnitine (Takahashi & Ueda, 1995), however, the gene encoding this enzyme was not determined.

#### Other carnitine derivatives

Carnitine is used in nutritional supplements, energy drinks, to replace carnitine lost during dialysis, and in treatments for carnitine uptake disorders. Because D-carnitine is inhibitory to uptake and metabolism in mammals, these industries have searched for cost-effective methods to synthesize the L-carnitine enantiomer. One strategy for L-carnitine synthesis is to start with a more easily synthesized enantiomeric precursor, like

L-carnitine nitrile or L-carnitine amide, in order to decrease the cost and streamline the production of L-carnitine. A bacterium isolated from the soil, DSM 6230 (no taxonomic classification has been published) metabolizes L-carnitine amide into L-carnitine and ammonia by a novel enzyme L-carnitine amidase (Kula & Joeres, 1993; Ulrich & Kula, 1994). The identified carnitine amidase is highly specific for L-carnitine amide and the D-enantiomer inhibits the enzyme and cannot be used as a substrate (Kula *et al.*, 1996). Another compound that can be metabolized to form carnitine is carnitinenitrile. A nitrilase from *Corynebacterium*, carnitinenitrile hydrolase, converts D- or L-carnitinenitrile into its corresponding D- or L-carnitine and ammonia (Kakayama, August 1991). Norcarnitine is a derivative of carnitine that has a diethylamino group instead of trimethylamino group, can be used as a sole carbon and nitrogen source in *P. putida* (Kleber *et al.*, 1978). The biological importance of these carnitine related enzymes has not yet been examined, but the findings suggest that there is either enzymatic flexibility in some of the carnitine metabolic enzymes, or that there are additional, naturally-occurring carnitine-like compounds in the environment.

### **1.2.9. Conclusions and Future Directions**

Bacteria import, synthesize, and metabolize carnitine through various pathways that have different physiological effects. Our understanding of carnitine transport and metabolism is derived from studying extracellular or facultative intracellular bacteria and examining how carnitine is obtained from either the environment or within an animal host. However, there are a number of important questions that remain to be addressed

related to carnitine-dependent transcriptional regulation, the ecological roles of carnitine, the role of carnitine in obligate intracellular bacteria, and its importance during non-pathogenic interactions like symbioses.

In relation to obligate intracellular pathogens, we know surprisingly little. For instance, to date, no spirochetes have been identified to use carnitine, but the causative agent of syphilis, *Treponema pallidum*, is predicted to harbor a carnitine transporter (Saier & Paulsen, 2000; Smajs *et al.*, 2005). With *T. pallidum* only having a thousand genes (Fraser *et al.*, 1998), carnitine transport may be important survival in certain environments and for establishing infection. Furthermore, it is tempting to speculate that intracellular pathogens and symbionts can likely use carnitine based on the role of carnitine-fatty acid transport systems in the mitochondria, particularly in light of the endosymbiotic source of these organelles. As such, one might expect a role for carnitine import in the Rickettsiales.

Beyond pathogens and symbionts, the impact of environmental metabolism of the osmoprotectants glycine betaine and dimethylsulfoniylpropionate (DMSP) has been reasonably well studied (reviewed in (Curson *et al.*, 2011; Welsh, 2000)), but carnitine has not received much attention in relation to its contributions outside of animal infection and likely deserves additional scrutiny. For instance, the source of carnitine detected in the environment (Warren, 2013a; Warren, 2013b) is unknown and we know very little about its half-life as a soluble compound in the environment or the flux rate of carnitine in any environment. A priori, one would assume an important role for carnitine metabolism, both aerobic and anaerobic, during animal decomposition on land or in

marine environments. In the deep oceans in particular carnitine utilization from fish and marine mammal carcasses might represent an important pathway to scavenge all available nutrients in this harsh environment.

Finally, we know almost nothing about the mechanisms by which carnitine is bound and detected to mediate transcriptional regulation. Further investigations into CdhR-like, CaiF-like, and the predicted carnitine-sensing TetR-family regulators in Gram positives will be needed to understand how ligand detection is accomplished and converted into regulation of gene expression. Of particular import will be understanding the binding pocket of these regulators to determine if they maintain the cation- $\pi$  binding aromatic cage that typifies the known quaternary amine binding proteins crystalized to date. From an evolutionary perspective, CdhR likely arose after gene duplication from a GbdR-like ancestor, while the Gram positive TetR-family proteins are reasonably similar to the choline binding transcription factor BetI. Thus, characterization of any of these quaternary amine binding transcription factors will provide crucial understanding of ligand binding and, by homology, present likely binding residues in their respective paralogs. Beyond direct ligand-sensing transcription regulators, we do not know of any two-component systems or chemotaxis regulators that sense and response to carnitine. The GlrA/GlrK system may represent the actual carnitine-sensing input that impinges on CaiF to establish carnitine-sensitive gene induction. Additionally, many bacteria have been shown to chemotax towards the quaternary amines DMSP and glycine betaine (Miller *et al.*, 2004; Seymour *et al.*, 2010; Stocker & Seymour, 2012), and therefore any

discovery of chemotaxis towards carnitine will further our understanding of its role in bacterial biology.

#### **1.2.10. Acknowledgements**

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### 1.2.11. Figures

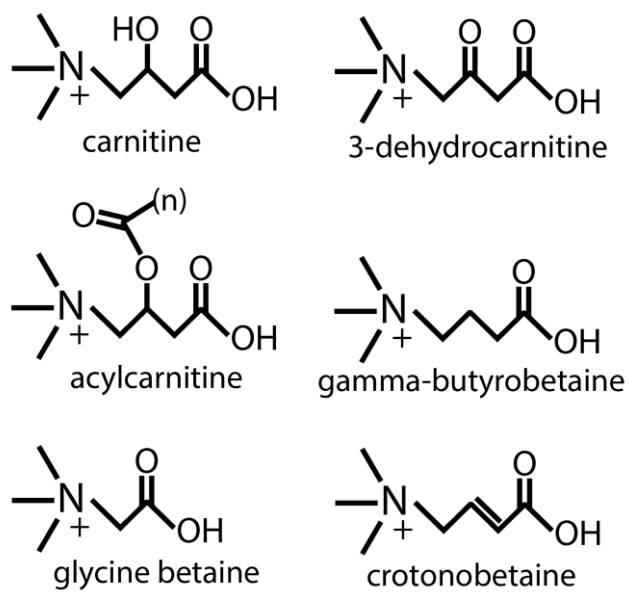
**Figure 1. Structures of carnitine and related compounds discussed in this review.**

**Figure 2. Diagram of the multiple pathways for carnitine metabolism in bacteria.**

Based on Uanschou et al. (2005). Metabolic steps described in this review are noted with numbered circles: (1) acylcarnitine hydrolase, HocS, ACH, EC 3.1.1.28; (2) gamma butyrobetaine hydroxylase, GBBH, EC 1.14.11.1; (3) oxidoreductase CntAB, EC 1.14.13.-; (4) carnitine racemase, EC 5.1.-.-; (5) L-carnitine dehydrogenase, L-CDH, EC 1.1.1.108; (6) D-carnitine dehydrogenase, D-CDH, EC 1.1.1.108; (7) beta-keto acid cleavage enzyme, BKACE, EC 3.-.-.-; (8) No ATP, spontaneous decarboxylation; (9) Nitrilase, EC 3.5.5.1; (10) Carnitine amidase, EC 3.5.1.73; (11) Carnitine methylase. Note that not all enzymatic steps have full EC descriptors at this time.

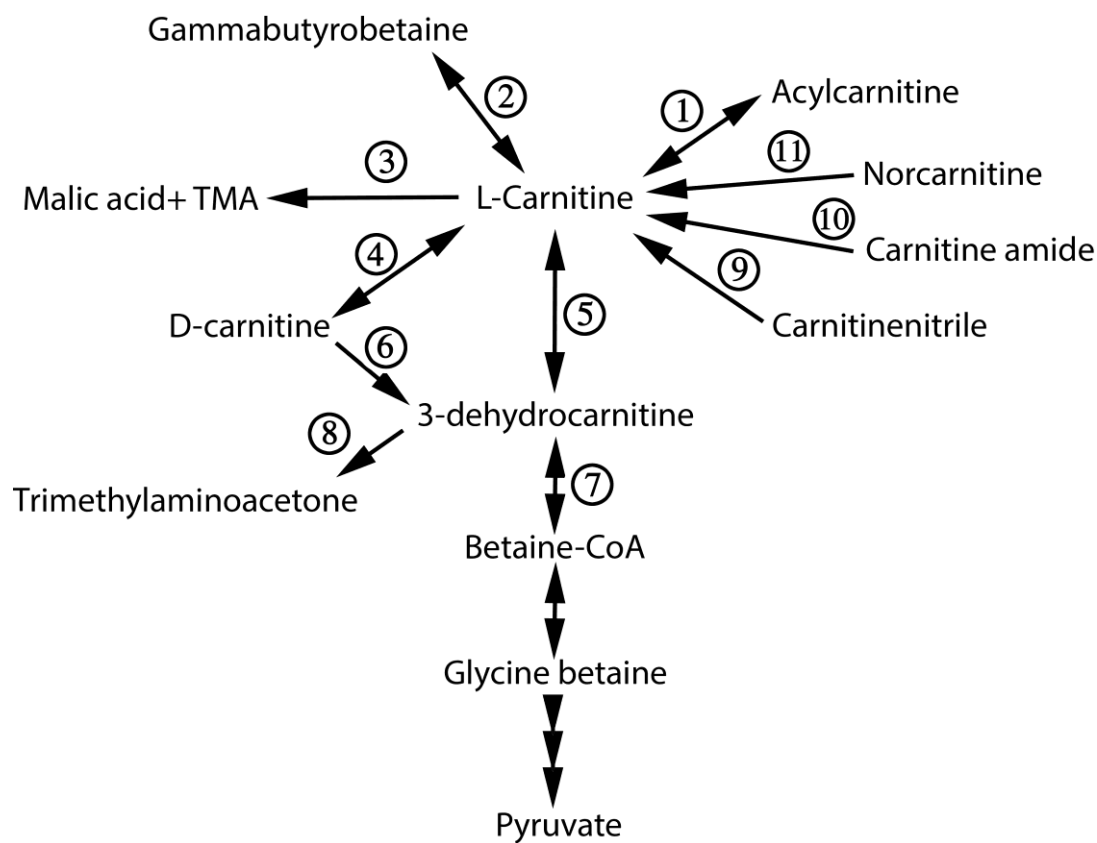
**Figure 3. Diagram of the Gamma butyrobetaine-Crotonobetaine-Carnitine cycle.**

Primarily based on Elssner et al. (2001), with additions based on Bernal et al. (2008) and Cánovas et al. (2003). GBB – gamma-butyrobetaine, CaiA - crotonobetaine reductase, CaiB - CoA transferase, CaiC - betaine:CoA ligase, CaiD - enoyl-CoA hydratase, FixABCX - electron transfer flavoproteins. Electron movement is denoted by  $e^-$ .

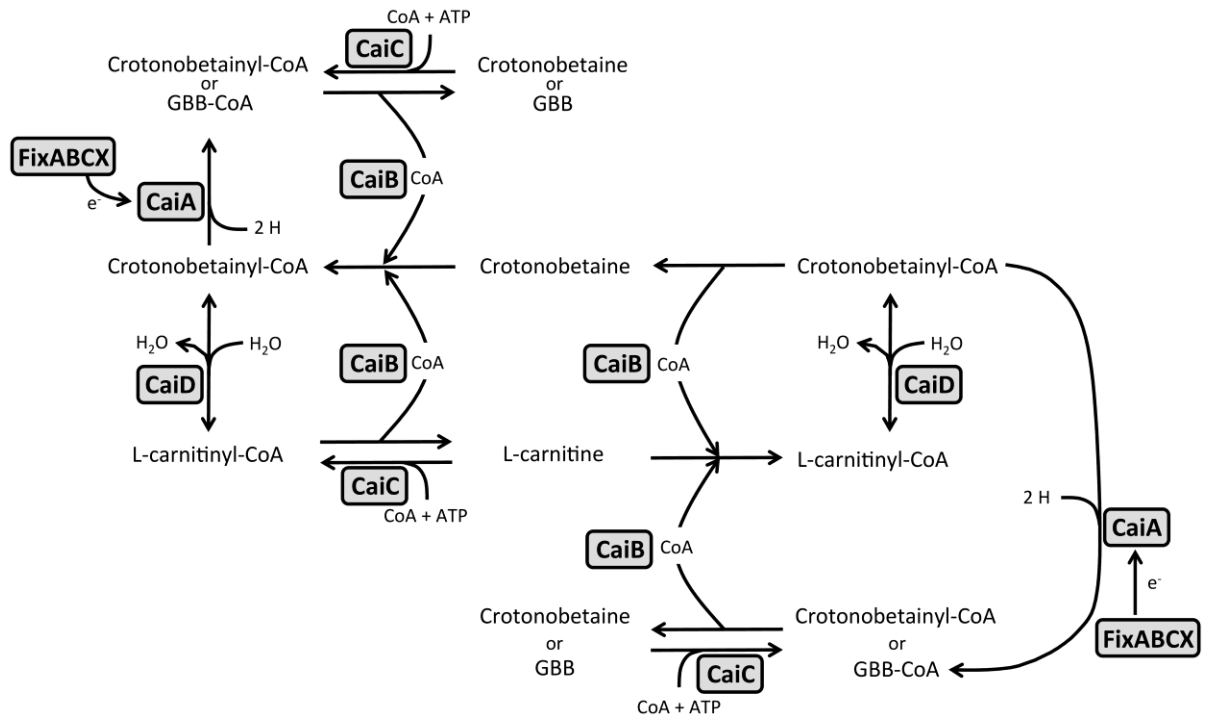


**Figure 1. Structures of carnitine and related compounds**





**Figure 2. Diagram of the multiple pathways for carnitine metabolism in bacteria**



**Figure 3. Diagram of the Gamma butyrobetaine-Crotonobetaine-Carnitine cycle**

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## **1.3 Catabolism of host-compounds during extracellular bacterial infections**

### **1.3.1 Abstract**

Efficient catabolism of host-derived compounds is essential for bacterial survival and virulence. While these links in intracellular bacteria are well-studied, such studies in extracellular bacteria lag behind, mostly for technical reasons. The field has identified important metabolic pathways, but the mechanisms by which they impact infection and in particular, establishing the importance of a compound's catabolism versus alternate metabolic roles has been difficult. In this review we will examine evidence for catabolism during extracellular bacterial infections in animals and known or potential roles in virulence. In the process, we point out key gaps in the field that will require new or newly adapted techniques.

### **1.3.2 Introduction**

To successfully cause infection, a bacterium must incorporate and metabolize molecules derived from the host. General maintenance and expression of virulence factors are minimal requirements, while efficient catabolism and resultant growth can lead to successful colonization and improved evasion of immune-mediated clearance. Here we will use the strict definition of catabolism: degradation of a compound yielding energy [Russell and Cook, 1995]. When we view bacterial catabolism in this way, the most straightforward analogy is to view the host as the growth medium [Brown et al., 2008]. This paradigm, while it's been around for 135 years and has seen intermittent resurgence [Garber, 1960; Pasteur, 1878], has seen many contributions within the last

two decades. Armed with the powerful techniques of modern bacteriology, solving the problems of in vivo bacterial catabolism, growth, and resultant virulence should be easy, right?

In one sense the answer is, undoubtedly, yes. In the case of intracellular pathogens, the eukaryotic host cells generate the correct 'growth medium' in the infected compartment. Experiments in tissue culture, presuming the correct cell type and growth condition, can very closely mimic the experience of the bacteria in a similar cell within the whole organism. These in vitro systems benefit tremendously from a few handy simplifications: (i) one or a few host cell types, (ii) high bacteria to host cell ratio, and (iii) facile monitoring and manipulation of both sides of the interaction. For many intracellular pathogens, their tractability has led to a reasonably good understanding of in vivo catabolism and its connection to virulence, particularly for *Legionella*, *Listeria*, *Salmonella*, and *Mycobacterium*, which have been well reviewed previously [Eisenreich et al., 2010] and will not be repeated here. Instead, this review focuses on extracellular bacterial pathogens; the little that is known, the vast areas that are not, and some thoughts about how to address the current unknowns. Our principle goal is to discuss what is known about the catabolic pathways governing in vivo growth and survival.

For extracellular bacteria, the primary technical issue can be boiled down to one thing: not knowing the growth medium. What looks to be straightforward turns out to be fraught with problems. We have certainly made important headway by using overly-simplified models, but we are left with a wide gap between these models and reality. We lose the dynamic, homeostatic nature of the extracellular niche when we remove this

niche from the organism or reconstitute it from parts [Smith, 2000]. Such in vitro characterizations of the extracellular milieu lack dynamics, while in vivo experiments are plagued with the problems readily overcome when studying intracellular pathogens: (i) numerous host cell types, tissues, and organs compared to a specific microenvironment within a specific cell type; (ii) overwhelming number of host cells to bacterial invaders; and (iii) poor spatial and temporal resolution of goings-on within the infection ([Smith, 2000] provides a thought provoking analysis of conceptual and technical problems associated with the study of catabolism in vivo). These issues do not make identifying important metabolic pathways overly difficult, rather they make dissecting the specific mechanism by which an implicated pathway alters in vivo growth, catabolism, and virulence incredibly hard. To bridge this gap in knowledge we need new techniques, greater incorporation of techniques from other fields, and a push towards mechanistic understanding of the importance of in vivo catabolism and specific catabolic pathways.

### **1.3.3. Assessing catabolism vs. alternate metabolic usage**

Catabolism has been extensively studied in vitro, where chemostats, microcalorimeters, and oxygen utilization measures have helped biochemists and microbiologists decipher the multiple roles of metabolized compounds during growth. Even within these in vitro systems, determining a compound's actual utilization requires both growth measures and flux analyses to extract information about the role of a compound in energy production versus growth yield [Russell and Cook, 1995]. Overlaid onto the challenges of studying catabolism in vivo is the issue that many important



compounds have multiple roles within the cell. Amino acids can be catabolized as an energy source, but they also participate in protein synthesis, are interconverted to other amino acids, and can have other functions: proline is a useful osmoprotectant [Csonka and Hanson, 1991]; serine is used to synthesize phosphatidylserine and as part of the C1 acquisition cycle [Anthony, 2011; Geiger et al., 2010]; tryptophan feeds into formation of many secondary metabolites including virulence-related molecules like PQS produced by *P. aeruginosa* [Farrow and Pesci, 2007], etc. Amino acids are not alone in having multiple functions: host-derived carbohydrates can often be used to decorate pathogen surfaces and incorporated into exopolysaccharides, while lipids can impact efflux mechanisms and envelope stress. In this review, we have tried to catalogue the strength of evidence for catabolism itself versus alternate usage of host-derived compounds. We will examine major classes of compounds in the following subsections, which are summarized in Table 1.

#### **1.3.4. Carbohydrates**

Colonization of mucosal surfaces is a key step in pathogenesis for many opportunistic pathogens. The mucosal surface is dominated by mucins, large glycoproteins that coat mucosal surfaces to trap particles and infectious agents [Thornton et al., 2008]. Mucins are enriched in sialic acid, an anionic nine-carbon carbohydrate [Vimr et al., 2004], thus it is not surprising that many mucosal pathogens have acquired pathways for sialic acid utilization. Some of the best evidence for catabolism of carbohydrates as a major source of energy is utilization of sialic acid by intestinal

pathogens. Pathogenic species of *Vibrio* can be transmitted by the ingestion of contaminated food or water and subsequently colonize the host intestine. *V. cholerae* causes acute diarrheal disease and *V. vulnificus* results in gastroenteritis that can lead to septicemia in immunocompromised individuals. *V. vulnificus* carries the nan genes for sialic acid degradation on its core chromosome, whereas *V. cholerae* strains have the nan genes on the Vibrio Pathogenicity Island 2 (VPI-2) [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009]. In vitro, deletion of the nan genes in both species results in the inability to utilize sialic acid as a sole carbon and energy source and in vivo studies demonstrate colonization and survival defects in the intestine of infected mice [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009]. In vitro growth assays for both species reveal no toxic effects from sialic acid accumulation intracellularly or in the growth medium due to this defective catabolism, suggesting that the observed growth defects in vitro correlate with the colonization defect in the intestine. Jeong et al. postulate that sialic acid catabolism contributes to pathogenesis by contributing directly to multiplication (energy production and growth yield) and Almagro-Moreno et al. point out that it may serve as a carbon source in times of high competition when colonizing the intestine. Both groups concur that sialic acid catabolism as an energy source ensures survival during infection [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009].

As with many potential catabolic substrates, sialic acid has other uses for some pathogens that can cloud the interpretation of in vivo studies. Sialic acid can be used to sialate lipids or exopolysaccharides as a means of immune evasion by bacterial pathogens [Vimr et al., 2004]. Deletions in genes encoding sialidases or sialic acid transporters have

been shown to cause colonization and survival defects in non-typeable *Haemophilus influenzae*, the major causative agents of middle ear infections [Jurcisek et al., 2005], as well as respiratory tract infections by *Streptococcus pneumoniae* [Marion et al., 2011]. In these studies, while sialic acid utilization as a catabolic substrate is possible, alterations in sialylation are also a potential mechanism. In addition, it is critical to separate catabolism from incorporation. It is possible that while sialic acid is broken down as an energy source by some of these pathogens, its importance may be for supplying carbohydrate substrates for sugar-intensive biosynthetic processes like capsule synthesis or biofilm formation. Degradation of sialic acid requires an initial investment of ATP and produces acetate and ammonia during partial metabolism even prior to the catabolic steps that would lead to energy production [Vimr et al., 2004]. The resultant ammonia could function as a nitrogen source, while the acetate may be important in a more global sense because acetate, and its co-regulated acetyl-CoA and acetate phosphate pools, impact lysine acetylation [Verdin and Ott, 2013]. Lysine acetylation has been implicated in global regulation of central metabolism and multiple catabolic pathways [Wang et al., 2010]. Consequently, such metabolic byproducts can impact catabolism of other substrates indirectly.

The carbohydrates deoxyribose and fucose have been implicated as catabolic substrates for pathogenic strains of *Escherichia coli*. *E. coli* is a Gram negative bacterium that is a normal inhabitant of the human intestinal flora. Pathogenic strains can be transmitted through fecal-oral or fecal-ureter route where they can colonize the intestine or the urinary tract resulting in extreme diarrhea or urinary tract infections, respectively.

For deoxyribose, many pathogenic *E. coli* contain the *deoK* operon encoding proteins for deoxyribose catabolism, allowing these organisms to use deoxyribose as a carbon source [Bernier-Febreau et al., 2004; Jonsen et al., 1959]. Strains incapable of deoxyribose catabolism have defects in intestinal colonization and are outcompeted by those strains capable of deoxyribose catabolism [Martinez-Jehanne et al., 2009]. It is known that deoxyribose is present in the intestinal mucus, the colonization defect of the *deoK* mutant is clear, and the presence of the *deoK* gene on one of the pathogenicity islands underlines its contribution to virulence, however, the evidence for direct catabolism is not strong. It is well documented that the gut is populated with a variety of bacteria and that metabolite sharing is a common phenomenon. Freter's nutrient theory states that in order for invading bacteria to compete and thrive in the intestine it must be able to more efficiently utilize a limiting nutrient better than resident bacteria [Freter et al., 1983]. Although *E. coli* can utilize fucose as a carbon source [Hacking and Lin, 1976], they do not contain genes encoding fucosidases [Hoskins et al., 1985], which cleave fucose from glycans. But by being in close proximity with the gut commensal *Bacteroidetes thetaiotamicron* and other strains, *E. coli* can capitalize on *B. thetaiotamicron*'s fucose cleavage by importing and catabolizing released fucose [Salyers et al., 1977; Xu et al., 2003]. In a bovine rectal colonization model, wild type *E. coli* O157:H7 out-competed a fucose catabolic mutant, *fucAO* (fucose aldolase and oxido-reductase respectively) exhibiting the importance of fucose utilization for *E. coli* colonization and maintenance of the population [Snider et al., 2009].

*E. coli* in vitro gene expression studies showed that genes involved in catabolism of gluconate and other sugars were highly induced when cells were grown in intestine-like conditions, 50% mouse cecal mucus. Gluconate was shown to be the preferred nutrient in vitro through mutation in *edd*, which encodes a 6-phosphogluconate dehydratase, which catalyzes a key step in gluconate entry into the Entner-Doudoroff pathway. In a mouse cocolonization model, the *edd* mutant had a defect in initiation and maintenance of intestinal colonization [Chang et al., 2004]. The gluconate catabolism was also shown to be important for intestinal colonization of *V. cholerae*, as the *edd* mutant failed to colonize the intestine in an infant mouse model [Patra et al., 2012].

#### **1.3.5. Amino Acids**

We previously remarked on the pleiotropic roles of amino acids within the cell. This integration with the whole metabolome often makes concrete conclusions about amino acid catabolism difficult. The best evidence for catabolic utilization comes from *Campylobacter jejuni*, for which serine is a preferred carbon and energy source [Leach et al., 1997]. *C. jejuni* is commensal to avian species but is spread to humans through the consumption of contaminated food and causes acute gastroenteritis. Mutations in L-serine deaminase (*sdaA*), which converts serine to pyruvate, lead to major *C. jejuni* colonization and survival defects in both the avian and mouse intestines, and the mouse liver [Hofreuter et al., 2012; Velayudhan et al., 2004]. Likewise, in uropathogenic *E. coli* (UPEC), L-serine can also be catabolized as a sole carbon and energy source [Su and Newman, 1991]. For UPEC, L-serine utilization is important for colonization of the

bladder, as an *sdaAsdaB* double mutant was defective in colonization of the mouse bladder [Anfora et al., 2007]. The reaction catalyzed by serine deaminase directly produces pyruvate, strongly suggesting serine as a catabolic substrate. However, pyruvate is also a central player in anabolic reactions and while separable in vitro, in vivo attribution of mechanism is difficult. Also, given the additional product of the reaction, ammonia, the role of L-serine as a nitrogen source in these infection models cannot be wholly discounted.

As mentioned above, *C. jejuni* has a propensity to utilize amino acids within the intestines of mice and birds, rather than the dietary and mucin-derived carbohydrates that are often preferred by other intestinal pathogens [Velayudhan and Kelly, 2002].

Therefore, it is not surprising that *C. jejuni* genes responsible for amino acid catabolism, including aspartate and proline, have also been shown to be important in intestinal colonization [Guccione et al., 2008; Hofreuter et al., 2012]. Proline is also important during *Staphylococcus aureus* burn infections and abscess formation [Schwan et al., 1998]. In these studies, it is not easy to ascertain the role being played by proline, as the gene disruptions were made in the proline transporter. Proline can be transported into the cell and accumulated in the cytosol, where it can function as an osmoprotectant.

Accumulation of various host-derived osmoprotectants is important for a variety of infectious agents and infection sites [Sleator et al., 2001], therefore it is likely proline plays this role for *S. aureus*, though catabolism has not been ruled out.

Mekalanos' group took a top down approach when it came to identifying factors that contribute to the virulence of *V. cholerae*. Using a suckling mouse intestine model,

they paired individual LysR-type transcriptional regulator mutants with the parental strain and tested for in vivo fitness of the mutant compared to the wild type [Bogard et al., 2012]. Of the 38 LysR-family mutants tested, two mutants were impaired in colonizing the intestine, one of which was *metR*, the methionine biosynthesis regulator that also has a role in catabolism [Bogard et al., 2012]. Two MetR controlled genes that are particularly important for mouse intestine colonization are *metJ* which encodes a methionine repressor and *glyA1* which encodes serine hydroxymethyltransferase [Bogard et al., 2012]. GlyA1 is potentially interesting, as it controls the flux of glycine to pyruvate via serine, but also glycine biosynthesis via serine. Based on the evidence from *C. jejuni* infections mentioned previously, serine may be abundant in the intestine and could play a role in either the catabolic or anabolic reactions; particularly the later if glycine or other glycine precursors are limiting.

The concentrations of host-derived metabolites are substantially altered during bacterial infection, particularly near the site of infection [Beisel, 1975; Smith, 2000]. A dramatic case of such metabolic change occurs during chronic lung infections of people with the genetic disorder cystic fibrosis by the opportunistic pathogen *Pseudomonas aeruginosa* [Burns et al., 1998]. During these chronic infections, tissue damage, immune response, and bacterial activities sculpt the milieu, resulting in thick mucus plugs in airways that can be expectorated as sputum [Boucher, 2004]. *P. aeruginosa* catabolizes many amino acids within sputum from cystic fibrosis patients in vitro, but displays a preference for alanine [Palmer et al., 2007]. The importance of alanine catabolism was demonstrated in a chronic rat lung infection model, showing that *P. aeruginosa* mutants

incapable of converting alanine to pyruvate could not compete well against wild-type *P. aeruginosa* [Boulette et al., 2009]. Similar to the cases of serine catabolism in *C. jejuni* and UPEC, this is a deamination in *P. aeruginosa*, resulting in the formation of ammonia along with pyruvate. Therefore, while loss of alanine catabolism is among the likely mechanisms driving the mutant phenotype, the impact of pyruvate-dependent anabolic pathways and the role of the released ammonia need to be considered.

### 1.3.6. Other compounds

Glycerol can be phosphorylated and incorporated into glycolysis and is also a critical contributor to phospholipid synthesis. In *Borrelia burgdorferi*, the causative agent of Lyme Disease, deletion of the glycerol catabolism gene *glpD*, which converts glycerol to dihydroxyacetone phosphate (DHAP), results in clear replication defects within the adult tick vector and nymph stage, while the deletion did not have any effect on growth or survival within the mouse model of infection [He et al., 2011; Pappas et al., 2011]. Deletion of *glpD* with the rest of the *glpDFK* operon resulted in a robust growth defect within the tick [He et al., 2011], likely because deletion of the entire operon limits glycerol incorporation into phospholipids in addition to elimination of a shunt of glycerol into glycolysis [Pappas et al., 2011].

L-lactate can be the sole energy source and is the preferred carbon source for the opportunistic dental and endocardial pathogen *Aggregatibacter actinomycetemcomitans* (Aa), even in the presence of high-yield compounds such as glucose or fructose [Brown and Whiteley, 2009]. Like many bacteria, Aa exists in multispecies communities and is



often found in conjunction with oral streptococci, which produce L-lactate as a catabolic product. *Streptococcus gordonii* and an *Aa* *lctD* (NAD independent L-lactate dehydrogenase) mutant co-inoculated into a mouse thigh abscess model was used to show that lactate catabolism in *Aa* is important in vivo for establishing polymicrobial infections [Ramsey et al., 2011].

The conversion of pyruvate to lactate by lactate dehydrogenase (ldh) is also an important part of NAD<sup>+</sup> regeneration in fermentative systems, which allows the continued operation of glycolysis. An example can be found in *Enterococcus faecalis*, a pathogen that can cause endocarditis, bacteremia, urinary tract infections and meningitis. *E. faecalis* has two *ldh* genes and when both are deleted there is a defect in colonization and persistence of the bacterium in an intravenous mouse model infection in the liver and kidney [Rana et al., 2013]. While NAD<sup>+</sup> also functions in other metabolic processes, the flux required by glycolysis suggests that the *ldh* activity in *E. faecalis* plays its main role in enabling robust glycolysis.

### **1.3.7. The importance of catabolism and its link to virulence**

Catabolism of host-derived compounds is necessary for successful infection. These compounds may be derived directly from the host or by scavenging compounds released by other pathogenic or commensal bacteria [Freter et al., 1983]. The studies described above have determined metabolic pathways important for bacterial survival and growth within the host, but in most cases, direct evidence for catabolism contributing to the in vivo phenotype is limited (well described in [Vimr, 2013]). From the most human-

centric standpoint, the exact mechanism is not critical to drive therapeutic development – efficacy and safety trump mechanism. However, we think that understanding the direct mechanism may lead to better future therapies by suggesting synergistic targets to inhibit with the appropriate drug cocktail. For example, if serine was truly a major energy source for a pathogen, combining the serine pathway inhibitor with an inhibitor of the next most important energy source would likely boost efficacy. However, if the importance of serine is to provide a ready supply of glycine or contribution to C1 or C2 biosynthetic units, a better strategy would be to target alternate pathways that produce the limiting intermediate. Therefore, a more complete understanding of the nature of the metabolic deficiency during infection will benefit future antimicrobial therapeutic development.

Determining the link between catabolism and virulence can be difficult. It is obvious that eliminating catabolism of host-derived compounds decreases the pathogenicity of the bacterial population. On the other hand, microbiologists often look at virulence in terms of production of anti-host products termed virulence factors, while catabolic pathways important for survival would fall under the term virulence determinant.

Under the heading of virulence determinant, we can certainly include all of the metabolic pathways discussed in this review. Each genetic mutation reduced the survival or growth of the bacteria and thus contributed to virulence in the model discussed. While virulence determinants can work indirectly, all of the bacterial species mentioned here also produce secreted virulence factors. Most of these are transported through the general or type III secretion systems and target host cells or specific pathways to boost

pathogenesis by increasing host damage and decreasing immune clearance. In the simplest sense, producing virulence factors requires energy and therefore are dependent on catabolic pathways. However, it is interesting to note two things. First, secreted proteins are generally less energy intensive than membrane or cytosolic proteins [Smith and Chapman, 2010] and consequently metabolically cheaper. Second, secreted virulence factors are a good investment during nutrient stress, as many described virulence factors, particularly extracellular enzymes transported by the general secretion system, can also be thought of as nutrient requisition systems [Rohmer et al., 2011], providing amino acids (proteases), sugars (glycosidases), nucleic acids (nucleases), and lipids, glycerol, and polar headgroups (lipases). The direct regulatory links between specific catabolism and virulence factor production are less readily apparent. However, it is very clear that, globally, the cell's nutrient and energy status strongly impacts virulence factor production, reviewed in [Poncet et al., 2009].

#### **1.3.8. Methods: current and a call for renewed and newly-adapted techniques**

Most studies of bacterial catabolism have not started as such. With some exceptions described here, catabolic pathways have often been identified during medium or high-throughput screens to identify mutants with altered in vivo survival. Moderate throughput has generally relied on direct counts of surviving colonies, often in competition with wild type cells. High-throughput techniques that have been particularly successful are in vivo expression technology (IVET) [Slauch et al., 1994], signature-tagged mutagenesis (STM) [Lehoux and Levesque, 2000], and transposon insertion

sequencing (TnSeq) [van Opijnen et al., 2009], all of which offer ways to assess large numbers of mutants simultaneously during infection. While generally useful, there are caveats associated with these methods of identification of catabolic pathways. One complication is that there are a variety of available carbon sources in a host – many at very high concentrations – so the contribution of any one carbon source may be relatively small, particularly for catabolically versatile bacteria.

Direct labeling: In the very simplest sense, we want to identify what compounds bacteria are eating in the host to provide them with energy, carbon, and nitrogen. There are direct and indirect ways to do this. A classic and still very useful method is to track the fate of radiolabeled substrates. While simple in vitro, many technical issues crop up in vivo. In general, a substrate is fed or injected into the animal and conversion of the substrate into radiolabeled CO<sub>2</sub>, acetate, or some other catabolic product is monitored. By comparing to an uninfected animal one can determine the proportion of compound utilized during infection. However, practically this is made very difficult for two reasons. First, host metabolism changes during infection, alterations due to bacterial processes are difficult to separate from changes due to the way the host changes its metabolism to fight the pathogen [Beisel, 1975]. Second, there is the gut microbiota, which by sheer numbers, provide a huge catabolic reservoir to convert substrates of interest. This leaves direct labeling useful mainly in two cases: gnotobiotic mice and compartmentalized substrates. While the former eliminates or reduces issues with gut metabolism, the later takes advantage of the fact that many compartments and organs maintain stable pools of compounds that are not rapidly converted or moved from the compartment. Examples

include particular proteoglycans in the joints, certain glycosphingolipids in the brain, and choline in the lung and brain. Other compounds that can be studied with this technique are those that are not catabolized by the host, such as L-carnitine, which while utilized in the carnitine/acylcarnitine shuttle system, is not catabolized by host cells [Peluso et al., 2000], although its anaerobic breakdown by the gut flora shows important links to cardiovascular health [Koeth et al., 2013].

**Stable isotope probing:** Stable isotope probing has been exceptionally useful for the study of catabolic processes in microbial ecology [Neufeld et al., 2007]. Briefly, an organism catabolizing a heavy-isotope substrate will incorporate the isotope into their DNA and the DNA of an otherwise ‘light’ organism will shift towards ‘heavy’. This shift is assessed with cesium gradient ultracentrifugation followed by fractionation. This technique falls victim to the same issues as direct radiolabeling, but the cesium gradient method allows discrimination of the source of the DNA by specific nucleic acid hybridization techniques. This technique, however, directly assesses the incorporation of label into DNA, an anabolic process. While these building blocks can be derived from catabolic end products and siphoned from catabolic intermediates, it is not a direct marker of catabolism.

**Using transcriptional evidence to understand catabolism:** One of the best ways to understand the range of possible food choices for the bacteria (but not their relative importance) is to look at the genes whose expression is induced in response to the host. As with the rest of bacterial catabolism in the host, the intracellular pathogens have yielded much more compelling transcriptional stories than their extracellular counterparts

at the site of infection. For extracellular pathogens, the staggeringly abundant host RNA has generally made sensitive measurement of the bacterial transcriptome difficult. There are some exceptions, where bacterial abundance or localized infections have allowed *ex vivo* transcriptomics [Bielecki et al., 2008; Chaffin et al., 2012; Larocque et al., 2005], although this field is changing rapidly with many host RNA subtraction techniques and massively parallel sequencing. Therefore, many host-pathogen systems are now sufficiently sensitive to determine the transcriptome of the bacterial population during infection.

A particularly useful application of these techniques would be to couple transcriptomics and metabolomics during infection in order to determine what compounds the bacteria senses and simultaneously measure the fate of the related metabolites. In most infection sites, the bacteria may not be able to exceed host homeostasis for many metabolites, therefore simultaneous analysis of the local transcriptome of the host may help determine compounds that the host senses it must make more of, essentially responding to a small decrease by making or transporting more of a given compound.

While global transcriptomics has its place, often we are asking questions about specific pathways or genes. In these cases, sensitive and quantifiable reporters are critical tools for analysis. The current generation of reporter genes works well for microscopic evaluation (fluorescent proteins) and high levels of expression (*lux* and *lacZ*). While these are both quantifiable under optimal conditions, a better proposition might be to use the power of reporter genes in combination with the sensitivity of radiodetection. One

potential method we have been discussing is to use the SNAP and CLIP proteins (NEB) as reporters, with increasing the sensitivity and specificity by labeling with radioactive substrates instead of fluorescent or biotin-linked substrates. A strain carrying one of the reporters as a translational fusion to the gene of interest and the other to a control gene would allow relative expression values to be sensitively determined by SDS-PAGE of the labeled tissue homogenate followed by autoradiography.

### **1.3.9. Conclusions**

Bacterial nutrition is a critical component of understanding infection and developing effective treatments. Part of this nutrition is utilization of compounds as energy sources, i.e. catabolism. Here we have provided some glimpses of specific compounds used by pathogens, but we're confident that the current work only scratches the surface of important host-derived compounds. The challenge for the field is to generate methods or new systems to determine the metabolic role of individual compounds for the infecting bacteria.

### **1.3.10. Acknowledgements**

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### 1.3.11. Tables

**Table 1. Summary of studies on extracellular pathogens implicating catabolism of host-derived compounds during animal infection**

Bacteria	Disease	Catabolite	Reference
Gram positive			
<i>Enterococcus faecalis</i>	Endocarditis, UTI, bacteremia, meningitis	Lactate	Rana et al. (2013)
<i>Staphylococcus aureus</i>	Skin infections, endocarditis, pneumonia, meningitis, bacteremia	Proline	Schwan et al. (1998)
<i>Streptococcus pneumoniae</i>	Pneumonia, meningitis, endocarditis, pericarditis, bacteremia	Sialic acid	Marion et al. (2011)
Gram negative			
<i>Aggregatibacter actinomycetemcomitans</i>	Periodontitis, infective endocarditis	Lactate	Ramsey et al. (2011)
<i>Borrelia burgdorferi</i>	Arthritis, Lyme	Glycerol	Pappas et al. (2011), He et al. (2011)
<i>Campylobacter jejuni</i>	Gastroenteritis, Guillain-Barré syndrome	Serine	Velayudhan et al. (2004)
		Proline	Hofreuter et al. (2012)
		Aspartate	Guccione et al. (2008)
		Serine	Anfora et al. (2007)
<i>Escherichia coli</i>	Gastroenteritis, UTI, neonatal meningitis	Deoxyribose	Martinez-Jehanne et al. (2009)
		Fucose	Snider et al. (2009)
		Gluconate	Chang et al. (2004)
<i>Haemophilus influenzae</i>	Otitis media, pneumonia, meningitis, bacteremia	Sialic acid	Jurcisek et al. (2005)
<i>Pseudomonas aeruginosa</i>	Lung infection, keratitis, UTI, bacteremia, folliculitis, endocarditis	Alanine	Boulette et al. (2009)
<i>Vibrio cholerae</i>	Acute diarrhea	Methionine	Bogard et al. (2012)
		Sialic acid	Almagro-Moreno et al. (2009)
		Gluconate	Patra et al. (2012)
<i>Vibrio vulnificus</i>	Gastroenteritis, septicemia	Sialic acid	Jeong et al. (2009)



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## **1.4. Carnitine transport**

### **1.4.1. Background**

In order for nutrients to get into the cell and for waste products to exit they must cross cytosolic membrane. Gram-negative bacteria have an additional membrane, the outer membrane that is semi-permeable and slows the diffusion of small hydrophilic molecules while inhibiting the passage of large and charged molecules [1, 2]. The outer membrane has transmembrane protein channels called porins that can allow passage of specific or nonspecific cargo and have many functions, one of which is to allow entry of nutrients and solutes [3, 4]. The cytoplasmic membrane acts as a semi-permeable barrier that prevents passive leakage of the cytosolic components but does not completely inhibit small or nonpolar molecules from diffusion, like oxygen or carbon dioxide. Large, hydrophilic, or charged molecules must be transported in and out of the cell.

Transport of nutrients into the cytoplasm occurs via simple diffusion, facilitated diffusion, or active transport. Simple diffusion is the movement of small, hydrophobic as nonpolar molecules directly across the membrane. Facilitated diffusion moves larger, polar, charged, and hydrophilic molecules into the cell using membrane proteins that span the cytosolic membrane. Both simple and facilitated diffusion are typically put in the same category of passive transport because neither requires energy and the molecules move from a higher concentration to a lower concentration or are driven by the electrochemical gradient of the cell [5]. However, active transport requires energy in order to translocate molecules and is often coupled with ATP hydrolysis or actively maintained ion gradients [5].

Carnitine is a polar zwitteronic compound that cannot pass through cell membrane and is actively transported using either ATP hydrolysis, ion motive force, or driven by a substrate:product antiporter. Many bacteria utilize and benefit from carnitine transport by using it as osmoprotectants, inducers of virulence factors, or as sole carbon, nitrogen, and energy sources [6], as discussed earlier in this section.

#### **1.4.2. ATP-Binding Cassette transporter**

ATP-binding cassette transporters, ABC transporters for short, are conserved through all domains of life [7] and have been present in every species studied as of 2006 [8]. The majority of characterized eukaryotic ABC transporters export substrates out of organelles or the cell, whereas most bacterial ABC transporters import substrates [9]. ABC transporters are composed of four core domains that span the lipid membrane and drive translocation of solutes by ATP hydrolysis. The core transporter forms a pore which ligand is transported into the cell, and is composed of two transmembrane domains (TMD) that bind ligand and two nucleotide binding domains (NBD) that bind and hydrolyze ATP. The core forms inward and outward facing configurations: the inward conformation has the TMD open to the cytoplasm, and the outward conformation has the TMD open to the periplasm [10]. ABC transporters also have substrate binding proteins; periplasmic in Gram-negative bacteria, or tethered to the cell membrane in Gram-positive, which binds ligand and delivers it to the TMD [7, 9].

ABC importers are divided into three classes or types. Type I and Type II transporters differ in that- Type I have an inward facing TMD and fewer transmembrane domains,



whereas Type II have more transmembrane domains, an outward facing TMD. Additionally, the interaction between the substrate binding protein (SBP) and the core transporter is weakened upon binding of substrate in Type II transporters [11, 12]. A recently categorized ABC transporter is Type III or ECF for energy-coupling factor. Type III transporters consist of the TMD-NBD and an S component instead of the SBP [11]. The S component binds substrates that are typically metal ions and vitamins [13].

#### **1.4.3. Components of ABC transporters**

Bacterial import of molecules via an ABC transporter requires a substrate binding protein that binds the substrate for delivery to the core transporter TMD-NBD. The SBP is either tethered to the cell membrane by an N-terminal lipid anchor or is covalently attached the TMD, as in Gram-positive bacteria. In Gram-negative bacteria, the untethered SBP is periplasmic [14, 15] and is in stoichiometric excess compared to the core components to increase the probability of substrate import. A good example of this is the *E. coli* maltose import system, which has 30 fold more SBP than TMD-NBD [16]. To date, all SBPs have the same tertiary structure with two lobes joined by a hinge. The SBP is in an open conformation until substrate binding when the lobes close around the substrate, described by the “venus fly trap” model [17]. Each SBP binds one substrate molecule at a time and delivers it to the TMD [18]. Ligand specificity of a SBP is not necessarily limited to one substrate, and some SBPs have multiple ligands; notable examples are OpuC in *B. subtilis* (carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine) [19,

20], LivJ in *E. coli* (leucine, isoleucine, and valine) [21], and CbcX in *P. aeruginosa* (choline and glycine betaine) [22].

The transmembrane domain of the ABC transporter spans the cytoplasmic membrane and is composed of two homodimers or heterodimers. Although each dimer typically has 6 alpha helices, 10-20 alpha helices have been reported [11]. TMD's have one or two substrate binding sites, although the two binding site configuration is more common [23]. A crystal structure of the *E. coli* BtuCD TMD revealed a 16 amino acid helix-turn-helix EAA motif, also known as the L loop, is conserved and located in the cytoplasm [24, 25]. This EAA motif that interfaces with the NBD and this interaction is likely how NBD conformational changes driven by ATP hydrolysis are transduced to the TMD [14]. Similar to the SBP, TMDs can bind and interact with multiple SBPs, which is observed in *P. aeruginosa* CbcWV [22] and *Salmonella typhimurium* HisQMP [26].

Energy required to drive import and export is generated from ATP hydrolysis. NBDs are highly conserved and are homologous throughout the family with seven domains: aromatic, Walker A, Walker B, ABC signature domain (LSGGQ), and Q, D, and H loops [7]. Two NBDs are required for each transporter complex and both must be functional for ATP hydrolysis and transport of substrates [9].

#### **1.4.4. Mechanism of action**

Import by ABC transporters begins with the SBP binding to its ligand and interacting with the TMD causing a conformation change, which is thought to initiate binding of ATP to the NBD [7]. The two NBDs each bind a magnesium-ATP, which

catalyzes dimerization, wherein the TMDs switch between the inward and outward conformation (or outward to inward depending on type of transporter). This interaction, ATP binding, and switching of the TMDs, is thought to transmit a signal to the SBP to release the substrate into the middle of the TMDs [9]. ATP hydrolysis takes place, releasing ADP, inorganic phosphate, and substrate resulting in what is called the “power stroke”, all of which resets the NBD and TMD for the next cycle of import [9, 12].

#### **1.4.5. Carnitine and ABC transporters**

Gram-positive and Gram-negative bacteria use ABC transporters to import carnitine for multiple uses: osmoprotection, cryoprotection, barotolerance, bile tolerance, and as a carbon, nitrogen, and energy source [6]. The components of the *P. aeruginosa* carnitine ABC transporter are located in two separate operons: the TMD and NBD are in the *cbcXWV* operon, and the SBP, *caiX*, is encoded in the carnitine catabolism operon. *cbcV* encodes the NBD and the TMD encoded by *cbcW* is predicted to have six transmembrane alpha helices, resulting in a total of 12 helices in the membrane spanning component upon homodimerization [27]. The SBP *caiX* is in the first gene in the carnitine catabolism operon and imports carnitine, acetylcarnitine, and butyrylcarnitine. The molecular details of how CaiX binds its ligands are not known but may be similar to the *B. subtilis* OpuCC protein, which binds carnitine, glycine betaine, and choline [20]. The cleft of OpuCC has an aromatic girdle composed of four tyrosine residues that interact with the trimethylammonium through cation- $\pi$  and hydrophobic interactions and

the carboxyl group is bound by hydrogen bonding to the amino acids glutamine, serine and asparagine within the binding pocket [20].

#### **1.4.6. Betaine/choline/carnitine transporters (BCCT)**

BCCT transporters, as the name would suggest, transport the compatible solutes choline, carnitine, and glycine betaine, all of which are quaternary amine compounds. More recently the substrate specificity has been broadened to include other compatible solutes: proline betaine, acetylcholine, and dimethylsulphoniopropionate [28]. Structurally, BCCTs typically have 12 transmembrane domains with the N- and C-termini extending into the cytosol. The driving force behind transport of substrate is either proton motive force, sodium motive force, or less commonly by substrate: product antiport, including a quaternary amine carnitine:γ-butyrobetaine antiporter. The most widely studied carnitine BCCTs are the CaiT orthologs from *E. coli* and *Proteus mirabilis*. CaiT is a substrate:product antiporter that imports carnitine during anaerobic conditions when glucose and a final electron acceptor are absent [29]. Under those conditions, carnitine and its metabolite crotonobetaine work as final electron acceptors and are both converted to gamma butyrobetaine, which is exported as the product in the antiporter system [29]. Recent studies demonstrate that CaiT binds carnitine or γ-butyrobetaine, which is similar to the substrate binding protein OpuCC (mentioned above). CaiT binds the positively charge amine head group using cation-π and van der Waals interactions and the carboxyl tail by hydrogen bonds to amino acids in the pocket [30].

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## **1.5. Regulation of bacterial gene expression**

In order for bacteria to be able to prosper and survive in their environment, they need to be able to precisely control the expression level and timing of critical genes. *P. aeruginosa* PAO1 has a large genome that consists of 5,570 predicted open reading frames that encode systems for exceptional metabolic diversity [1]. It would not be advantageous for bacteria to transcribe and translate unnecessary genes, as it uses the energy and resources of the cell. Therefore, the cell tightly regulates gene expression and protein function in many ways. So, as part of this dissertation centers on two different transcription factors and how they contribute to carnitine regulation, the focus of this section will be on initiation of transcription, one of the steps for gene regulation.

### **1.5.1. Initiation of transcription**

Transcription is a concerted effort between multiple proteins to make a RNA transcript. Chromosome size, compactness, DNA binding proteins, and epigenetics all play a role in initiation of transcription, but the required protein for transcription is the multisubunit RNA polymerase (RNAP). In order for RNAP to initiate transcription at a promoter, it must first interact with a sigma factor subunit to form the RNAP holoenzyme. *P. aeruginosa* PAO1 has 24 sigma factors that recognize and bind to the -10 and -35 elements (or functionally equivalent elements) of the promoter DNA, which are located 10 and 35 bases upstream from the transcriptional start site [2, 3]. The -10 site interacts with domain 2 and the -35 site interacts with domain 4 of the RNAP sigma 70 subunit, the housekeeping or primary sigma factor. Once the RNAP is bound, the DNA is



denatured and forms an open promoter complex. The sigma factor dissociates and allows for elongation of the transcript to occur [4].

### **1.5.2. Transcription factors: inhibiting or promoting initiation**

Transcription factors (TF) are proteins that couple environmental or internal signals and to gene expression. By binding specific DNA sequences TF activate or inhibit transcription by recruiting or blocking RNAP from binding promoter regions [5]. TF are divided into families based on protein structure but typically have a sensing domain and an effector domain. Transcription factors generally are two-domain proteins (ligand and DNA binding domains), meanwhile two component regulatory systems consist of a membrane bound kinase and a cytoplasmic response regulation that interacts with the DNA to regulate gene expression [6]. In order to be able to respond to a wide array of environmental signals, bacteria need many different regulatory proteins and in *P. aeruginosa* PAO1 there are over 400 transcriptional regulators [7]. TFs can act as activators or repressors, with some having dual activity.

Activators bind DNA sequences called enhancers and are classified by the mechanism they use to recruit RNAP. Class I activators bind upstream of the promoter region and bind to the alpha subunit C-terminal domain ( $\alpha$ CTD) of RNAP. Class II activators have an enhancer site that overlaps the promoter -35 element. Lastly, class III activators bind enhancers near or overlapping the promoter, resulting in a conformational change of the promoter DNA to an orientation that is favorable to RNAP binding [5].

Repressors inhibit transcription by impeding RNAP from binding the promoter. There are three basic mechanisms of repression: (i) repression by steric hindrance, typically by binding the promoter region and blocking RNAP from binding the promoter, (ii) looping the DNA making the promoter site inaccessible, and (iii) by modulating an activator [5].

Many promoters are controlled by two or more TFs that work in concert to tune gene expression to match environmental signals. Multiple activators can work together to recruit RNAP, or an activator can inhibit a repressor allowing for a second activator to initiate transcription. The regulatory circuit combinations are vast [5].

### **1.5.3. The AraC transcription factor family**

Proteins of the AraC transcription factor family, named after the arabinose responsive regulator in *E. coli*, are present in Gram-positive and Gram-negative bacteria and function as activators or repressors [8]. The family is defined by a conserved 99 amino acid C-terminal domain [8], which consists of two helix-turn-helix motifs that bind to the major grooves of the DNA. Each monomer contacts one of the two half sites in the DNA binding sequence, typically oriented in direct repeats or inverted repeats [9]. While the N-terminal domain functions in ligand binding and dimerization, the sequence and structure of the N-terminus is not conserved, such that there are a number of subfamilies based on the identities of the N-termini. [10, 11]. The first 18 amino acids of the N-terminal domain is the arm, which interacts with the DNA binding domain in the absence of ligand and holds the protein in a conformation not amenable to dimerization. On the

hand, in the presence of ligand the arm undergoes a conformational change to fold over the ligand [12, 13]. Both domains are linked together by a flexible linker that tethers the two domains together. The length, flexibility, and structure of the linker play a role in the regulation between the two domains [14, 15].

The mechanism for AraC regulation of the arabinose operon *araBAD* in *E. coli* has been established and is termed the “light switch” mechanism (figure 1). In the absence of arabinose, the N-terminal arm binds the C-terminal DNA-binding domain allowing for a conformation that promotes the DNA-binding domains to bind the half sites  $I_1$  and  $O_2$ , looping the DNA and inhibiting transcription [16]. When arabinose binds, it is more favorable for the arm to dissociate from the DNA-binding domain and interacts with the dimerization domain and ligand [17, 18]. It is then energetically favorable for the DNA-binding domains to bind  $I_1$  and  $I_2$  instead of looping the DNA and this leads to transcriptional induction [16, 17]. This mechanism has been studied extensively with AraC but is not ubiquitous for all AraC TFs.

Schleif and colleagues constructed a chimeric protein consisting of the AraC ligand binding domain and LexA DNA binding domain that showed the LexA DNA binding domain bound its operator in an arabinose dependent manner, suggesting that the two domains can function independently from one another, at least in the case of AraC [19]. The N-terminal domain provides the ligand and sensory specificity for the regulator. The variability in the N-termini among the AraC-family TFs allows for control and regulation of carbon metabolism, stress responses, and virulence [8]. Rob, MarA, and SoxS are a few AraC TFs that respond to stresses such as antibiotic, oxidative stress, or organic

solvents, respectively [20]. Carbon metabolism of arabinose in *E. coli* [21] is the most studied AraC-family TF and is described in detail above [22]. Lastly, AraC-family TFs can regulate virulence. ToxT regulates the toxin-coregulated pilus and cholera toxin, two virulence factors controlled by environmental stimuli. Bicarbonate increases the affinity of ToxT to the promoters while unsaturated fatty acids decrease DNA binding affinity [23, 24].

#### **1.5.4. The GATase-1 AraC transcription factor subfamily**

The varied AraC N-terminal domains are divided into subfamilies, one of which is a focus in our laboratory: the Type 1 glutamine amidotransferase- like domain (GATase-1, pfam00117). Glutamine amidotransferases are enzymes that have a catalytic triad of cysteine, glutamine, and histidine that remove an ammonia group from glutamate and transfer it to a substrate. The GATase-1 domain is conserved in some AraC TFs in *P. aeruginosa* and the members do not have a catalytic triad, and instead have a conserved cysteine residue at the nucleophilic elbow. The TFs CdhR and GbdR presented in this thesis belong to the GATase-1 AraC TF subfamily and together allow detection of the charged amine compounds carnitine, glycine betaine (GB), and dimethylglycine (DMG).

*P. aeruginosa* GbdR, the glycine betaine-dimethylglycine regulator, has been studied to a much greater extent and regulates carbon metabolism and virulence [25]. Eukaryotic plasma membranes and lung surfactants contain significant amounts of phosphatidylcholine (PC) and sphingomyelin (SM). Phospholipase C (PlcH) enzymatically cleaves PC or SM to yield phosphorylcholine, which is dephosphorylated

by PchP [26, 27]. *In vitro* experiments show *plcH* and *pchP* transcripts were induced in the presence of surfactant- and are dependent on GbdR [25], and *in vivo* mouse lung pneumonia models show that PlcH reduces lung function [28]. Choline liberated from PC/SM is catabolized to GB and subsequently to pyruvate. This pathway is primarily controlled by GbdR [29, 30] and serves multiple purposes for *P. aeruginosa* by generating an osmoprotectant [31], inducing *plcH* [25], and producing carbon, nitrogen, and energy sources [32]. Import of choline and GB is also partially regulated by GbdR [29, 30, 33].

The lesser known of the two GATase-1 TFs is CdhR, the carnitine dehydrogenase regulator. CdhR is divergently transcribed from the carnitine catabolism operon. Prior to the work presented here, our lab demonstrated that CdhR is required for growth on carnitine and is necessary for induction of the carnitine catabolism operon [22].

#### **1.5.5. Post-transcriptional regulation**

Regulation is complex and there can be many layers of control. As soon as a ribosome binding site (RBS), also known as the Shine-Delgarno sequence, of an mRNA exits the RNAP, translation can start, and in bacteria this can be concomitant with transcription. However, it is now appreciated that most translation occurs on free mRNAs [34]. With that being said, there is post-transcriptional regulation that often modulates stress responses and metabolic pathways [35].

RNA secondary structure can influence translation. Riboswitches are hairpin loop structures in the RNA 5' UTR that respond to environmental signals (temperature,

uncharged tRNAs, or metabolites) in a protein independent manner to modulate gene expression [36]. Typically genes that control metabolite synthesis or import are next to the riboswitch [36]. Riboswitches can activate but more often repress gene expression by forming a termination stem loop blocking or allowing RNAP access to the ribosome binding site [37]. Small RNAs (sRNA), usually small noncoding snRNA, are *cis*- or *trans*-encoded regulatory elements that bind RNA resulting in negative gene expression by inhibiting translation, or by promoting RNA degradation or cleavage. However, stimulation of gene expression can occur by sRNAs but is not as common. The sRNAs act by relieving secondary RNA structures that would normally inhibit ribosome binding but upon binding causes a conformational change stimulating translation [35]. Post-transcriptional regulation can also be controlled by proteins interacting with 5' UTR, affecting RNA stability and translation. In times of decreased nutrient availability or entry into stationary phase, the *E. coli* CsrA protein will bind RNA to modify gene expression of proteins involved in carbon metabolism and motility [38, 39].

#### **1.5.6. Other forms of regulation beyond RNA**

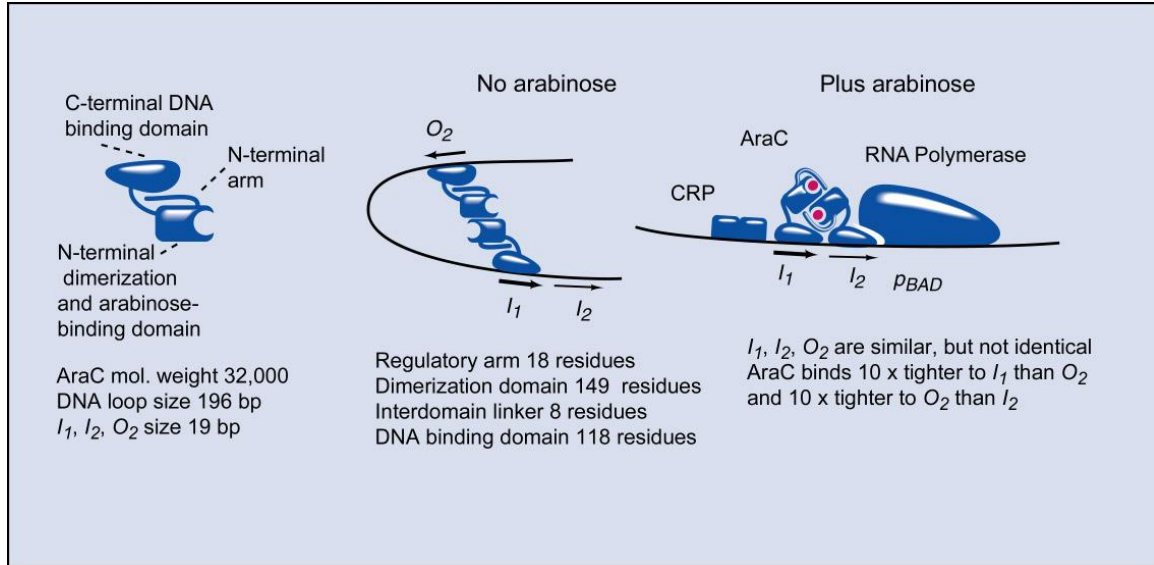
These other forms of regulation are vast and expansive subjects as well, but will not be discussed in detail. The experiments in this dissertation examine transcriptional regulation, with limited analysis of post-transcriptional regulation, and how they impact growth. Post-translational modification (PTM) occurs after protein translation, often in response to environmental changes or factors with more than 300 types known as of 2010 [40]. Enzymes chemically modify proteins allowing for quick and specific changes that

alter the proteins function, localization, and activity. One of the most common forms of PTM is the reversible process of phosphorylation. Specific proteins will have a phosphate group(s) added or removed to change their activity. Protein degradation is a very useful way to regulate processes. Bacterial pathogens *S. typhimurium* and *Listeria* have even found a way to hijack the host ubiquitin protein degradation system to degrade their effector proteins [40]. Some other common PTMs are glycosylation, acetylation, deamination, and many more, as reviewed in Ribet and Cossart [40].

### 1.5.7. Figures

**Figure 1. The regulatory region of the *araCBAD* genes.** O1, I1, and I2 are half-sites that a single subunit of AraC binds. When there is no arabinose, the AraC monomers bind to half sites O1 and I1 and cause the DNA to loop and inhibit transcription. In the presence of arabinose an AraC monomer switches from the O1 to the I2 half site resulting in a formation that allows transcription of the *araBAD* operon.





**Figure 1. AraC light switch mechanism <http://gene.bio.jhu.edu/>**

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## **CHAPTER 2:**

# **CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* GROWTH ON *O*- ACYLCARNITINES AND IDENTIFICATION OF A SHORT-CHAIN ACYLCARNITINE HYDROLASE**

Jamie A. Meadows<sup>1</sup> and Matthew J. Wargo<sup>1,2</sup>

<sup>1</sup> Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, VT, USA

<sup>2</sup> The Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT, USA

## 2.1. Abstract

To survive in varied environments, from host tissue to soil, opportunistic bacterial pathogens must be metabolically flexible and able to use a variety of nutrient sources. We are interested in *Pseudomonas aeruginosa*'s catabolism of quaternary amine compounds that are prevalent in association with eukaryotes. Carnitine and acylcarnitines are abundant in animal tissues, particularly skeletal muscle, and are used to shuttle fatty acids in and out of the mitochondria where they undergo  $\beta$ -oxidation. We previously identified the genes required for carnitine catabolism as the first four genes in the carnitine operon (*caiXcdhCAB*, PA5388-PA5384). However, the last gene in the operon, PA5384, was not required for carnitine catabolism. We were interested in determining the function of PA5384. Bioinformatic analyses along with the genomic location of PA5384 led us to hypothesize a role for PA5384 in acylcarnitine catabolism. Here we have characterized PA5384 as an L-enantiomer specific short-chain acylcarnitine hydrolase that is required for growth and hydrolysis of acetyl- and butyrylcarnitine to carnitine and the respective short-chain fatty acid. The liberated carnitine and its downstream catabolic product glycine betaine are subsequently available to function as osmoprotectants in hyperosmotic environments and induce transcription of the virulence factor phospholipase C, *plcH*. Furthermore, we confirmed that acylcarnitines from 2 to 16 carbon chain lengths, except for octanoylcarnitine (8-carbon), can be utilized by *P. aeruginosa* as sole carbon and nitrogen sources. These findings expand our knowledge of short-chain acylcarnitine catabolism and also point to remaining questions related to acylcarnitine transport and hydrolysis of medium- and long-chain acylcarnitines.

## 2.2. Introduction

Carnitine and acylcarnitines are quaternary amine compounds that are abundant in mammalian tissues and play important roles in eukaryotic metabolism (1). Carnitine facilitates the translocation of fatty acids into the mitochondrial matrix where they undergo  $\beta$ -oxidation to produce energy for the cell (2, 3). This translocation is dependent on the formation of an ester linkage between the fatty acid and the hydroxyl oxygen on carbon three; this esterified carnitine is referred to as *O*-acylcarnitine or, as we will use throughout this study, acylcarnitine. While there are no eukaryotic enzymes that catabolize carnitine (2), free carnitine can be utilized by some bacteria, including *Pseudomonas aeruginosa*, as a sole carbon, nitrogen and energy source (4, 5). The concentration of carnitine and acylcarnitines in humans range from 50  $\mu$ M in plasma to the low mM range in tissues, thus these compounds are potential sources of energy in colonization and infection (2, 6, 7). In *P. aeruginosa*, carnitine and its downstream catabolic product, glycine betaine, can function as osmoprotectants (8-10) and may be important for enhanced growth in hyperosmotic conditions, such as the environment of the lungs of people with cystic fibrosis and the urinary tract (11, 12). The generation of glycine betaine via carnitine catabolism also allows carnitine to induce expression of the virulence factor hemolytic phospholipase C, PlcH (8). During infection, PlcH can function to induce a proinflammatory response, suppress oxidative burst in neutrophils, degrade pulmonary surfactant, and increase endothelial cell death (13-18).

We previously identified the metabolic pathway responsible for carnitine catabolism in *P. aeruginosa* and determined that all but the last gene in the carnitine

catabolism operon are required for carnitine degradation (19). The last gene in the operon, *PA5384*, is immediately downstream of the carnitine dehydrogenase genes (CDH), but is not required for growth on carnitine (19). However, the genomic location of *PA5384* provided insight into its potential function. Uanschou *et al.* compared the genomic sequences of predicted CDH genes in Gram positive and Gram negative bacteria and determined that many of the CDH-containing operons had an unidentified esterase/lipase downstream of the CDH gene(s) (7). *PA5384* fits this pattern, as it is immediately downstream of *cdhB*, and is bioinformatically predicted to be a lipolytic enzyme (19, 20). The prediction made by Uanschou *et al.* that *PA5384* and homologs encode an acylcarnitine hydrolase, has not been directly tested. Previous work has briefly biochemically characterized an acylcarnitine hydrolase from an *Alcaligenes sp.* (21) that is used as a diagnostic tool for measuring acetylcarnitine in serum. However, this study provided no genetic identification associated with the purified hydrolase (22). Acylcarnitine catabolism has been studied in *Pseudomonas putida* (23) but it was not known if *P. aeruginosa* can hydrolyze acylcarnitines or employ them as a sole carbon and nitrogen source.

In this study we demonstrate that *P. aeruginosa* can utilize acylcarnitines as sole carbon, nitrogen, and energy sources. We also demonstrate that hydrolysis of acylcarnitines enables osmoprotection via production of carnitine and the downstream catabolite glycine betaine; improving cell survival in hyperosmotic environments and inducing the expression of the virulence factor PlcH. In addition, we characterize the function of the *PA5384* enzyme and determine its role in acylcarnitine metabolism.



## 2.3. Materials and Methods

### 2.3.1. Strains and growth conditions

*P. aeruginosa* PA14 (24), PAO1 (25), and derivative strains were maintained on Pseudomonas Isolation Agar (PIA, Difco) plates or in LB broth with gentamicin added when necessary at 50  $\mu\text{g ml}^{-1}$  or 40  $\mu\text{g ml}^{-1}$ , respectively. *E. coli* NEB5 $\alpha$  was maintained on LB plates or in LB broth supplemented when necessary, with gentamicin at 10  $\mu\text{g ml}^{-1}$  or 7  $\mu\text{g ml}^{-1}$ , respectively, or in LB broth with 100  $\mu\text{g ml}^{-1}$  kanamycin when needed.

To assess growth on acylcarnitines, *P. aeruginosa* cultures were pre-grown overnight at 37°C on a rotary wheel in 3 ml of MOPS minimal medium with 20 mM pyruvate and 5 mM glucose added as carbon sources (26). When necessary, gentamicin was added to these minimal media cultures at 20  $\mu\text{g ml}^{-1}$ . Cells from these overnight cultures were added to MOPS media without a nitrogen source or MOPS media with 20 mM of the specified carbon source in 48 well plastic culture dishes at a final OD<sub>600</sub> of 0.05. These plates were shaken at 170 rpm for 24 hours at 37°C for carnitine and acylcarnitines with 2-14 carbon chain lengths (designated 2C, 4C, ...16C) or at room temperature for palmitoylcarnitine due to micelle formation of palmitoylcarnitine at 37°C. Growth was assessed by OD<sub>600</sub> on a Synergy 2 BioTek plate reader. Measurement of growth at OD<sub>600</sub> was not possible for palmitoylcarnitine due to the optical interference from the micelles that formed; therefore, triplicate serial dilutions were plated onto PIA and incubated for 24 hours to determine CFU ml<sup>-1</sup>. The suppliers for the compounds are as follows: L-carnitine, L-acetylcarnitine, and D,L-palmitoylcarnitine are from Sigma, L-butyrylcarnitine and D-acetylcarnitine are from Crystal Chem., D,L-hexanoylcarnitine,

D,L-decanoylcarnitine, D,L-laurylcarnitine and D,L-myristoylcarnitine are from Biotrend Chemicals and D,L-acetylcarnitine is from Tocris Bioscience. The D,L-octanoylcarnitine was purchased from two different suppliers, Tocris Bioscience and Biotrend Chemicals. Biotrend Chemicals synthesize their compounds onsite in Germany, whereas Tocris Bioscience purchased the D,L-octanoylcarnitine from a producer in the United Kingdom (ascertained through personal communication).

### **2.3.2. Octanoylcarnitine competition assay**

To determine if octanoylcarnitine inhibits growth on other acylcarnitines, *P. aeruginosa* PA14 was pre-grown overnight at 37°C on a rotary wheel in 3 ml of MOPS media with 20 mM pyruvate and 5 mM glucose added as carbon sources. Cells from the overnight culture were added at a final OD<sub>600</sub> 0.05 to MOPS media with 10 mM of either acetylcarnitine or decanoylcarnitine as the carbon source. Octanoylcarnitine (Tocris Biosciences or Biotrend Chemicals) was added at 5 mM and the cultures incubated at 37°C overnight. Growth was assessed by OD<sub>600</sub> on a Synergy 2 BioTek plate reader.

### **2.3.3. Expression and deletion constructs**

The *PA5384* deletion construct was generated in the pMQ30 plasmid (27) and the deletion in *P. aeruginosa* PA14 was made by recombination as described previously (28, 29). Briefly, the upstream and downstream regions of the *PA5384* gene were amplified by PCR from pMW79 using the primers: PA5384-GOI-A 5'-aagcttGCCTGACCTTCCAGGACAT-3', PA5384-SOE-A 5'-

aagtacgaaggcgactcgaccatggGGCGAGGCAGGGTTCTAT-3', PA5384-SOE-B 5'-  
ccatggtcgagtcgccttcgtacttCCGGAGACAGCGGATACTT-3', and PA5384-GOI-B 5'-  
gaattcATTGCCCTGGACCTACCTG-3'. Lower case letter sequences in the above  
primers represent complementary regions for splice overlap extension or engineered  
restriction sites. The splice overlap extension PCR product was cloned into the pCR-  
Blunt vector (Invitrogen), excised with HindIII and EcoRI, and cloned into similarly cut  
pMQ30 by *E. coli*-based cloning as described previously to generate pMW86. Donor *E.*  
*coli* S17/λpir carrying the *PA5384* deletion construct (pMW86) were mated with *P.*  
*aeruginosa* PA14 and single crossover mutants selected for growth on PIA plates with  
gentamicin. Recombinants were verified by PCR. Double crossover events were selected  
by growth on 5% sucrose LB plates with no NaCl (28, 30).

The *PA5384* expression vector (pJAM8) was made by amplifying *PA5384* from  
the plasmid pMW79 (19) using primers 5384ET30a F 5'-  
ggatccGCTGCGAAGTATCCGCTGTCT-3' and 5384ET30a R 5'-  
aagcttCTATTCGCCTGGCTGGTG-3'. This product was cloned into the pCR-Blunt  
vector using the Zero Blunt cloning kit (Invitrogen), with subsequent transformation and  
plasmid preparation as described above. The resulting plasmid was digested with HindIII  
and BamHI-HF (NEB) and the ~1kb fragment was extracted from an agarose gel and  
ligated into the similarly digested pET-30a expression vector (Novagen). The newly  
assembled plasmid, pJAM8, was transformed into chemically competent T7 Express  
Competent *E. coli* (NEB C2566) and transformants selected on LB with kanamycin.

#### 2.3.4. Trans complementation of *PA5384::tn* mutant strains

To complement the PAO1 *PA5384::Tn* mutant strains (31) (MJ206 and MJ207), we placed the *PA5384* gene at the *attTn7* site under control of the promoter regulating the native operon. The promoter of the carnitine catabolism operon (*PA5388*) was amplified from plasmid pMW79 using primers 5388promfor-5384F 5'-TAGCggtaccGGTTGAGGTTGCGCAGCC-3' and 5388promfor5384R 5'-ATCAggatccCATCGGTCTCCCCTCGTG-3'. The 213 bp product was ligated into the pCR-zero blunt vector using the Zero Blunt cloning kit (Invitrogen), with subsequent transformation and plasmid preparation as described above. The resulting plasmid was digested with KpnI-HF and BamHI-HF (NEB) and the 213 bp fragment was extracted from an agarose gel and ligated into the similarly digested pJAM8 to generate pJAM61. The pJAM61 plasmid was digested with KpnI-HF and HindIII-HF and the ~1.2 kbp fragment was ligated into the similarly cut plasmid pUC18-mini-Tn7T-Gm (32) and transformed into chemically competent *E. coli* NEB5 $\alpha$ . Transformants harboring the newly generated plasmid, pJAM62, were selected in the presence of 7  $\mu\text{g ml}^{-1}$  gentamicin. The *PA5388* promoter fused to *PA5384* was integrated onto the chromosome of the PAO1 *PA5384::tn* mutant strains as previously described (18, 32). Briefly, the plasmids pJAM62 and pTNS2 (33) were co-electroporated into each of the PAO1 *PA5384::tn* mutant strains and transformants were selected on 50  $\mu\text{g ml}^{-1}$  gentamicin.

### 2.3.5. Expression and purification of PA5384

Overnight cultures of JM17, a T7 Express Competent *E. coli* (NEB) carrying pJAM8, in LB with 100  $\mu\text{g ml}^{-1}$  of kanamycin were inoculated into flasks containing 300 mL LB supplemented with 100  $\mu\text{g ml}^{-1}$  of kanamycin and grown to an  $\text{OD}_{600}$  of 0.3-0.4. The cultures were then induced with Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 hours at 37°C. Cells were collected by centrifugation and the pellet was resuspended in lysis buffer (B-PER, Thermo Scientific) at 4 ml per 1 g of cells. Halt<sup>TM</sup> protease inhibitor cocktail, EDTA-free 100X (Thermo) at 1X concentration, 150 mM NaCl, 2  $\mu\text{l ml}^{-1}$  lysozyme (50 units  $\text{ml}^{-1}$ , Thermo Scientific), and 2  $\mu\text{l ml}^{-1}$  DNase (2500 units  $\text{ml}^{-1}$ , Thermo Scientific), were added to the culture and incubated with gentle shaking at 37°C for 20 minutes. The lysate was clarified by centrifugation at 8,000 x g for 20 minutes at 4°C. The clarified lysate was applied to a HisPur cobalt-NTA column (Thermo), washed thoroughly with wash buffer #1 (10 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4), and wash buffer #2 (wash buffer #1 with 33 mM imidazole), and protein eluted off the column with elution buffer (70 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4). The elution fractions containing N-terminal hexahistidine PA5384, determined by SDS-PAGE gel electrophoresis, were pooled and dialyzed in a 20,000 MWCO Slide-A-Lyzer (Thermo Scientific) in 0.1 M potassium phosphate buffer pH 7.2 and stored at 4°C. The protein concentration was determined to be 37  $\mu\text{g ml}^{-1}$  by using bovine serum albumin standards in a Coomassie Plus – The Better Bradford<sup>TM</sup> Assay Kit according to Thermo instructions.

### **2.3.6. Acetylcarnitine hydrolysis assay**

An assay developed to detect acetylcarnitine for a diagnostic application previously described by Tomita *et al.* 2001 (22) was modified for the purpose of detecting enzymatic activity of the purified protein encoded by *P. aeruginosa* PA5384. The coupled assay of Tomita *et al.* was modified as follows. Purified 6xHis-PA5384 in 0.1 M potassium phosphate buffer pH 7.2 was added to the reaction mixture (0.1 M potassium phosphate buffer pH 7.2, 7 mM MgSO<sub>4</sub>, 50 mM KCl, 2.5 mM ATP, 0.5 M phosphoenolpyruvate, 0.3 mM NADH, 20 units ml<sup>-1</sup> acetate kinase (Sigma), 9 units ml<sup>-1</sup> pyruvate kinase (Sigma), and 4 units ml<sup>-1</sup> L-lactate dehydrogenase (Sigma)) in a 96 well-plate and incubated with shaking at 30°C (Synergy 2 BioTek) for 15 minutes. After this equilibration period, substrate (L-acetylcarnitine, D-acetylcarnitine, D,L-acetylcarnitine, or acetylcholine) was added to each well at 250 µM or 125 µM and incubated for an additional 3 hours at 30°C. During this incubation the absorbance was read at 340 nm every 3 minutes with each read preceded by 15 seconds of shaking.

### **2.3.7. Hydrolysis reactions for other acylcarnitines and detection of enzymatic activity**

The substrate specificity of PA5384 was determined by detecting free L-carnitine using a colorimetric L-carnitine assay (BioVision). The assay was performed as described by the manufacturer with the modifications described here. First, in a 96 well plate, 15 µl of 0.1 M potassium phosphate buffer pH 7.2 was added to each well of L-carnitine standard and brought to a final volume of 50 µl with the provided assay buffer. In a 96

well plate L-acylcarnitines 2C to 4C were added to a final concentration of 0.5 mM and D,L-acylcarnitines 6C to 16C were added to a final concentration of 1 mM in 0.1 M potassium phosphate buffer pH 7.2. The assay had technical triplicates and had more than three biological replicates for each carbon chain length. 0.5 µg of 6xHis-PA5384 was added to each well and incubated at 30°C while shaking for 15 seconds every 3 minutes for 3.5 hours. As a control, in triplicate, 0.1 M potassium phosphate buffer pH 7.2 and 0.5 mM acylcarnitine 2C to 4C or 1 mM 6C to 16C were added to the assay to detect signal from free carnitine present in these compounds. Assay controls according to BioVision's protocols were also performed with the modification of adding 0.5 µg 6xHis-PA5384 to the background control, which has no converting enzyme.

#### **2.3.8. PA5384-FLAG-tag and localization by western blot**

For western blot analysis, PA5384 was C-terminally FLAG-tagged via chromosomal integration by PCR amplifying the C terminus of *PA5384* with primers 5384Flag F 5'-ggtaccGAGCAGCCCCCTGGAGTTC-3' and 5384Flag R 5'-aagcttCTACTTATCATCATCATCCTTGTAATCCGGCTTCAGGTTCATCCTG-3' which contains the FLAG-tag. The 585 base pair product was cloned into the pCR-Blunt vector (Invitrogen) and excised with KpnI and HindIII. The excised product was cloned into similarly cut pMQ30 (27). Donor *E. coli* S17/λpir carrying the *PA5384*-FLAG construct, pJAM34, underwent conjugation with *P. aeruginosa* PA14 and single crossover mutants selected for growth on PIA plates with gentamicin. Recombinants were verified by growth in MOPS with 20 mM acetylcarnitine as a sole carbon source

supplemented with 20  $\mu\text{g ml}^{-1}$  gentamicin and by whole lysate western blots using anti-FLAG monoclonal antibodies (Sigma). The western blots were performed as follows: electrophoresis in 8% or 12% SDS-PAGE gel followed by protein transfer to a PVDF membrane (Immobilon-P Millipore). The membrane was blocked overnight in 5% nonfat dried milk in Tris buffered saline – Tween 20 (TBS-T: 150 mM NaCl, 50 mM Tris HCl, 0.1% Tween 20, 0.01% sodium azide, pH 7.6). The membrane was washed in TBS-T, then incubated for 1 hour with the anti-FLAG M2 monoclonal antibody (Sigma) or anti-SadB antibody (34) and washed in TBS-T before detection with anti-mouse IgG HRP secondary antibody (GE Healthcare) or anti-rabbit IgG IRDye 800CW (Odyssey). After washing with TBS-T, HRP was detected via enhanced chemiluminescence western blotting detection reagent (GE Healthcare) prior to exposing membrane to film.

*PA5384*-FLAG strains JM116 and JM117, and control JM141 were grown overnight to mid log phase in MOPS with 5 mM glucose, 20 mM carnitine and 15  $\mu\text{g ml}^{-1}$  gentamicin. JM141 is a control to account for nonspecific binding of the anti-FLAG monoclonal antibody to a random pMQ30 integrant (JM141 is with a single crossover integrant in *PA4921*, a gene irrelevant to the CDH pathway). Cells were collected by centrifugation at 8,000 x g for 10 minutes, and the supernatant was saved as the extracellular fraction. The pellets were resuspended in 20 mM Tris HCl pH 7.3 and lysed by repeated passage through a French Press at 9,000 PSI. The lysates were centrifuged at 8,000 x g for 10 minutes to remove non-lysed cells and large cellular debris. Separation of membrane from soluble fraction (cytoplasmic and periplasmic) was performed by ultracentrifugation at 100,000 x g for 30 minutes. The whole membrane fraction (outer



and inner membrane) was resuspended in 20 mM Tris HCl pH 7.3 and washed three times. The soluble fraction centrifuged at 100,000 x g three times to ensure no membrane contamination. The protein from the extracellular fraction was concentrated using a 10,000 MWCO Centriprep Centrifugal Filter (Millipore). The extracellular, soluble, and membrane fractions were then analyzed by western blot following the protocol above.

### **2.3.9. Osmoprotection assay**

Osmoprotection was determined by first growing *P. aeruginosa* PA14 WT and  $\Delta PA5384$  overnight at 37°C on a rotary wheel in 3 mL of MOPS medium with 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended to a final OD<sub>600</sub> of 0.05 in MOPS (which contains 50 mM NaCl) with an additional 800 mM NaCl, 20 mM glucose, and 0.2 mM carnitine or acetylcarnitine in 48 well plates. Cultures were grown at 37°C for 48 hours, due to the slow growth rate in a high salt medium, and growth was determined by OD<sub>600</sub>.

### **2.3.10. *plcH* promoter activity by means of *lacZYA* fusion**

Transcriptional induction from the *plcH* promoter was measured as previously described (35). Briefly, the *plcH-lacZYA* reporter construct, pMW22 (35), was transformed by electroporation into *P. aeruginosa* PA14 WT and  $\Delta PA5384$  and transformants selected for growth on gentamicin. Cells were grown overnight in MOPS with 20 mM pyruvate, 5 mM glucose, and 20 µg ml<sup>-1</sup> gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS with 20 mM pyruvate and 20 µg ml<sup>-1</sup>

gentamicin with the addition of 1 mM carnitine or acetylcarnitine for induction samples. Cells were induced for 5.5 hours at 37°C and  $\beta$ -galactosidase assays were performed according to the method of Miller (36).

#### **2.3.11. NPPC assay**

Phospholipase C (PLC) activity was measured by  $\rho$ -nitrophenylphosphorylcholine (NPPC) hydrolysis according to the method of Kurioka and Matsuda (37), as previously modified in our laboratory (18, 35). Briefly, cells were grown overnight in MOPS, 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended in MOPS with 20 mM pyruvate and 1 mM carnitine or acetylcarnitine and grown overnight at 37°C in a 48 well plate. Supernatants from these cultures were obtained and added to 2X reaction buffer (200 mM Tris-HCl pH 7.2, 50% glycerol, 20 mM NPPC) in a 1:1 ratio. Hydrolysis of NPPC was quantified by measuring absorbance at 410 nm using the extinction coefficient  $17,700 \text{ M}^{-1}\text{cm}^{-1}$ .

### **2.4 Results**

#### **2.4.1. *PA5384* is required for growth on acetylcarnitine.**

We previously demonstrated that the first four genes in the *caiXcdhCAB-PA5384* (*PA5388-PA5384*) operon (Fig. 1A) were critical for aerobic carnitine transport and metabolism (19, 38). *PA5384*, the last gene in the operon, was not required for carnitine catabolism (19). We hypothesized that *PA5384* may be associated with acylcarnitine catabolism based on its presence in the carnitine catabolism operon and bioinformatic

prediction of lipase/esterase function. To determine the function of *PA5384*, we made an unmarked deletion of *PA5384* ( $\Delta PA5384$ ) in *P. aeruginosa* PA14 and measured growth on acetylcarnitine. The *PA5384* deletion mutant was unable to grow on acetylcarnitine as a sole carbon source (Fig. 1B). To test whether *P. aeruginosa* could use either acetylcarnitine stereoisomer, we used both the L- and D-enantiomers of acetylcarnitine in a growth assay, and found that *P. aeruginosa* can only grow on the L-enantiomer of acetylcarnitine (Fig. 1 B). It has been shown that carnitine can be used as a sole nitrogen source (23) and we tested if acetylcarnitine can be used as a sole nitrogen source. *P. aeruginosa* PA14 can grow on acetylcarnitine as the sole nitrogen source whereas the  $\Delta PA5384$  deletion strain cannot (Fig. 1C).

We were unable to complement the acetylcarnitine growth defect of *PA5384* deletion strain using a high-copy constitutive plasmid (*PA5384* on the pUCP22 backbone), or using an arabinose-inducible high-copy system (*PA5384* under  $P_{BAD}$  control on the pMQ80 backbone). Therefore, to test if the growth phenotype in the *PA5384* mutant strain was due to mutation of *PA5384*, we constructed a *PA5388* (*caiX*) promoter fusion with *PA5384* ( $P_{caiX}$ -*PA5384*) and integrated it onto the chromosome at the *attTn7* site in two independent PAO1 transposon mutant strains. The two PAO1 transposon mutants, MJ207 and MJ206, carry a Tn5 transposon that confers resistance to tetracycline inserted into the *PA5384* coding sequence at base pairs 236 and 570, respectively (Fig. 1A) (31). Both strains carrying  $P_{caiX}$ -*PA5384* at the *attTn7* site (JM118 and JM119) were able to grow on acetylcarnitine as a sole carbon source (Fig. 1D). These data confirm the necessity of *PA5384* for growth on acetylcarnitine.

#### **2.4.2. PA5384 exhibits acetylcarnitine hydrolase activity.**

*P. aeruginosa* requires *PA5384* for growth on acetylcarnitine (Fig. 1). To determine if *PA5384* encoded an acetylcarnitine hydrolase, we expressed recombinant *PA5384* protein with an N-terminal hexahistidine tag (6xHis) and purified it by cobalt affinity chromatography and dialysis as described in the methods section. To measure acetylcarnitine hydrolase activity of *PA5384*, we adapted an enzymatic method designed to measure acetylcarnitine in serum (22), which uses four coupled enzymatic reactions to convert acetate abundance to an increase in NAD<sup>+</sup> concentration, resulting in decreased UV absorbance at 340 nm dependent upon acetate. We predicted that *PA5384* was an acetylcarnitine hydrolase that would hydrolyze acetylcarnitine to carnitine and acetate, and allow detection of acetate using the coupled reaction system. Using this assay we showed that *PA5384* has L-enantiomer specific acetylcarnitine hydrolase activity, as it hydrolyzes L-acetylcarnitine and not D-acetylcarnitine (Fig. 2). The specificity of *PA5384* was examined further by determining if the presence of the carnitine backbone of acetylcarnitine was necessary for hydrolysis. The inability of *PA5384* to hydrolyze acetylcholine, which is another quaternary amine containing ester, to choline and acetate suggests that the carnitine backbone is necessary for acetylcarnitine hydrolysis and that *PA5384* is not a general hydrolase (Fig. 2).

#### **2.4.3. PA5384 has specificity for short-chain acylcarnitines.**

As demonstrated above, *PA5384* is required for growth on acetylcarnitine and purified 6xHis-*PA5384* hydrolyzes acetylcarnitine in vitro. In the infection environment,

*P. aeruginosa* encounters a variety of acylcarnitines with different *O*-acyl chain lengths. To determine whether *PA5384* was required for growth on *O*-acyl chain lengths longer than acetylcarnitine we compared PA14 wild type to a  $\Delta PA5384$  mutant strain for growth on acylcarnitines of 2 to 16 carbon (2C to 16C) lengths. Here we show that *PA5384* is required for growth on short-chain acylcarnitines (2C and 4C), but not for growth on medium or long-chain acylcarnitines (Fig. 3A). Interestingly, *P. aeruginosa* cannot use octanoylcarnitine as a sole carbon source (Fig. 3A), nor does octanoylcarnitine inhibit growth on acetylcarnitine or decanoylcarnitine as the sole carbon source (data not shown). This observation will be addressed in the discussion section.

We sought to determine if the chain length specificity for the observed growth phenotype was due to enzymatic specificity of *PA5384*. To test in vitro hydrolysis of different acylcarnitines by *PA5384*, we measured L-carnitine release using a colorimetric assay for L-carnitine detection (BioVision). Each acylcarnitine from 2C to 16C chain length was assayed for free L-carnitine either in the presence or absence of purified 6xHis-*PA5384*. The samples with no *PA5384* enzyme added allowed for subtraction of contaminating free L-carnitine in the starting materials. The results show that *PA5384* can hydrolyze the short-chain acylcarnitines, acetyl and butyryl, but it has no activity on medium-chain and long-chain acylcarnitines (Fig. 3B). Based on the above L-carnitine assay and our genetic evidence, *PA5384* will now be referred to as HocS (Hydrolase of O-acylcarnitine, short-chains), with *hocS* being the corresponding gene name for *PA5384*.

#### **2.4.4. HocS is located in the cytoplasm.**

HocS was bioinformatically predicted to be in the cytoplasm using the PSORTb database v3.0 with a cytoplasmic score of 9.97, well above the accepted cut-off of 7.5 (39, 40). To confirm this prediction, we tracked the localization of a chromosomally incorporated C-terminally FLAG-tagged HocS. Cells with the *hocS-FLAG* allele were grown in carnitine to induce expression (19). Western blot analysis was performed on the extracellular, soluble, and insoluble fractions and HocS-FLAG was detected by use of an anti-FLAG monoclonal antibody (Fig. 4) compared to the untagged HocS control. A known cytoplasmic protein, SadB, was used as a control for cytoplasmic localization as well as cytoplasmic contamination in the extracellular and insoluble fractions using the anti-SadB antibody (34). Based on this data, HocS is most likely cytoplasmic, although this data does not conclusively exclude periplasmic localization.

#### **2.4.5. HocS is important for osmoprotection and induction of the virulence factor PlcH.**

Carnitine is known to be an osmoprotectant (8) and to induce phospholipase C activity (PLC) (8). The ability of HocS to liberate carnitine from short-chain acylcarnitines led us to predict its involvement in osmoprotection and PlcH activity under conditions where short-chain acylcarnitines were present. To determine if *hocS* has a role in osmoprotection we compared PA14 wild type to the *hocS* deletion strain for the ability to grow in the presence of 850 mM NaCl. Under these conditions carnitine functions as an osmoprotectant allowing for growth in wild type and  $\Delta hocS$  mutant strain (Fig. 5A).

Osmoprotection was also observed with acetylcarnitine as the osmoprotectant source in PA14 wild type but not in the  $\Delta hocS$  mutant strain, which had a three-fold decrease in growth compared to wild type (Fig. 5A). Choline is oxidized to glycine betaine, a potent osmoprotectant (9), and was used as control to show that deleting *hocS* did not compromise the choline catabolic pathway or general osmoprotection. These results support two conclusions. First, acetylcarnitine is not an effective osmoprotectant prior to hydrolysis, and second, that hydrolysis of acetylcarnitine by HocS can provide osmoprotection in hyperosmotic environments.

Release of carnitine by HocS activity would also be predicted to impact *plcH* transcription and PLC enzyme production by supplying glycine betaine for GbdR dependent induction of *plcH*. To test this prediction, we measured *plcH* transcription using a plasmid based *plcH-lacZ* reporter (pMW22) (35), and measured PLC enzymatic activity using the NPPC assay (37). Carnitine induced transcription of *plcH* as previously reported (35), in both the PA14 wild type and  $\Delta hocS$  mutant strains. However in the  $\Delta hocS$  mutant strain acetylcarnitine showed no *plcH* transcriptional induction whereas PA14 wild type showed robust induction (Fig. 5B). Activity of secreted PLC was determined for both PA14 wild type and  $\Delta hocS$  using the NPPC assay. A seven fold increase of PLC activity induced by acetylcarnitine was observed in PA14 wild type compared to  $\Delta hocS$  (Fig. 5C). These results demonstrate that *hocS* hydrolysis of acetylcarnitine is required for acetylcarnitine-dependent induction of *plcH* transcription and PlcH activity.

## 2.5. Discussion

In this study, we identified HocS (PA5384) as a short-chain acylcarnitine hydrolase that is required for *P. aeruginosa* to utilize acetyl- and butyrylcarnitine as sole carbon, nitrogen, and energy sources. Using a coupled enzymatic assay we show that HocS is specific for acylcarnitines and in conjunction with the L-carnitine assay we demonstrate that HocS specifically hydrolyzes short-chain acylcarnitines. The free carnitine generated can induce *plcH* transcription and result in induced PLC activity. *P. aeruginosa* can also utilize the rendered carnitine as an osmoprotectant to promote growth in hyperosmotic conditions. Through trans complementation we were able to show that *hocS* is required for *P. aeruginosa* grow on acetylcarnitine. We speculate that plasmid complementation was not successful due to the concentration of HocS compared to the other carnitine dehydrogenase proteins.

Acylcarnitine degradation has only been evaluated in *Pseudomonas putida* and *Pseudomonas fluorescens* (23, 41), which led us to inquire if *P. aeruginosa* can utilize acylcarnitines as sole carbon and nitrogen sources. In humans, acetylcarnitine is the most prevalent acylcarnitine intracellularly and in circulation but medium- and long-chain acylcarnitines are also present throughout the body, with long-chain acylcarnitines being the dominate acylcarnitine in bile (1). *P. putida* cannot utilize acylcarnitines from 2 to 8 carbon chain lengths but it is capable of using acylcarnitines 10 carbon chain lengths and longer as a sole carbon source (23). This led us to hypothesize that *P. aeruginosa* can utilize longer chain fatty acid length acylcarnitines as well. To the best of our knowledge, we are the first to show that *P. aeruginosa* can utilize acylcarnitines from 2 to 16 carbon



chain lengths as sole carbon and nitrogen sources (Fig. 1C and Fig. 3A). Interestingly, our data illustrate that *P. aeruginosa* cannot use octanoylcarnitine as a sole carbon source and octanoylcarnitine does not inhibit growth on either acetyl- or decanoylcarnitine. The failure to grow on octanoylcarnitine may be due to an inability to transport or hydrolyze octanoylcarnitine, either or both of which suggest that *P. aeruginosa* is specifically restricted in its use of octanoylcarnitine (Fig. 3A). We used two different sources of octanoylcarnitine that were manufactured in two different sites (United Kingdom and Germany) to rule out toxic contaminants and defective synthesis, so we think that this restriction is a biological one, although we do not know the mechanism governing this restriction. *P. fluorescens* strain IMAM on the other hand can hydrolyze L-octanoylcarnitine (41) but no study to date has shown growth on octanoylcarnitine as the sole carbon source in the pseudomonads.

Takahashi demonstrated, through crude purification, that an acylcarnitine hydrolase from *Alcaligenes* could effectively be employed to identify acetylcarnitine in biological samples (22, 42, 43). However, the gene responsible for the acetylcarnitine hydrolase activity in *Alcaligenes* was not determined. We successfully identified the gene and expressed the protein responsible for hydrolysis of short-chain acylcarnitines in *P. aeruginosa*, PA5384 (*HocS*), and determined that it is likely localized in the cytoplasm (Fig. 4). Data from the L-carnitine assay further illustrate that *hocS* only hydrolyzes the short-chain acylcarnitines acetyl- and butyrylcarnitine (Fig. 3B). Since *hocS* hydrolyzes only substrates with a carnitine backbone, it suggests that an accessible fatty acid alone is not sufficient to achieve hydrolase activity and that the hydrolase activity is specific to

the bond position on carnitine, which is further supported by the enantiomeric selectivity of the enzyme. These data and the ability of  $\Delta hocS$  mutant strain to grow on medium- or long-chain acylcarnitines as the sole carbon source suggest that *P. aeruginosa* possesses an alternate hydrolase(s) that can liberate carnitine from medium- and long-chain acylcarnitines.

An additional goal of this study was to determine if short-chain acylcarnitines have a physiological role in *P. aeruginosa* in addition to use as nutrient sources. The virulence factor PlcH is induced by phosphate deprivation and by the products of choline and carnitine catabolism; glycine betaine and dimethylglycine (8, 44, 45). As access to carnitine enables induction of *plcH*, we hypothesized that the free carnitine generated by HocS hydrolysis of short-chain acylcarnitines would provide a source of carnitine to induce *plcH*. The lack of induction in either *plcH* transcription or PLC activity in the  $\Delta hocS$  mutant in the presence of acetylcarnitine lead us to conclude that the carnitine generated from hydrolysis of short-chain acylcarnitines by HocS is sufficient to induce *plcH* expression (Fig. 5B-C).

The ability of *P. aeruginosa* to survive in hyperosmotic conditions by employing quaternary amine osmoprotectants has long been established (1, 8), such as choline-O-sulfate, carnitine, and glycine betaine. Even though acylcarnitines had not been evaluated in *P. aeruginosa* as osmoprotectants, our data shows that free carnitine generated by HocS hydrolysis acts as an osmoprotectant for *P. aeruginosa* in hyperosmotic conditions but that acetylcarnitine itself is not an osmoprotectant (Fig. 5A). In *Bacillus subtilis*, acetylcarnitine is a precursor to carnitine which is then used as an osmoprotectant (46). In

*Listeria monocytogenes*, acetylcarnitine functions as an osmoprotectant (47, 48), but these studies did not address if acetylcarnitine is catabolized to carnitine. *P. aeruginosa* has been shown to catabolize carnitine to glycine betaine in hyperosmotic environments (46). Short-chain acylcarnitines are a source of carnitine that can ultimately be converted to glycine betaine.

This study has expanded our knowledge on *P. aeruginosa* acylcarnitine catabolism. Similar to *P. putida*, *P. aeruginosa* can grow on acylcarnitines from 10 to 16 carbon chain lengths, but *P. aeruginosa* is additionally capable of utilizing acylcarnitines from 2 to 6 carbon chain lengths. *P. aeruginosa* hydrolyzes short-chain acylcarnitines using an L-enantiomer specific hydrolase we have described here, HocS. The liberated carnitine from short-chain acylcarnitines hydrolysis has multiple physiological impacts, including induction of virulence factors and providing access to osmoprotectants.

## **2.6. Acknowledgements**

We'd like to thank Dr. Heather Bean (University of Vermont) for technical advice and Dr. George O'Toole (Geisel School of Medicine at Dartmouth College) for generously providing us with the anti-SadB antibody. This project was supported by grants from the National Center for Research Resources (5P20RR021905-07) and the National Institute of General Medical Sciences (P20 GM103496 and P30 GM103532) from the National Institutes of Health.

## 2.7. Figures

### **Figure 1. *PA5384* is required for *P. aeruginosa* growth on L-acetylcarnitine.**

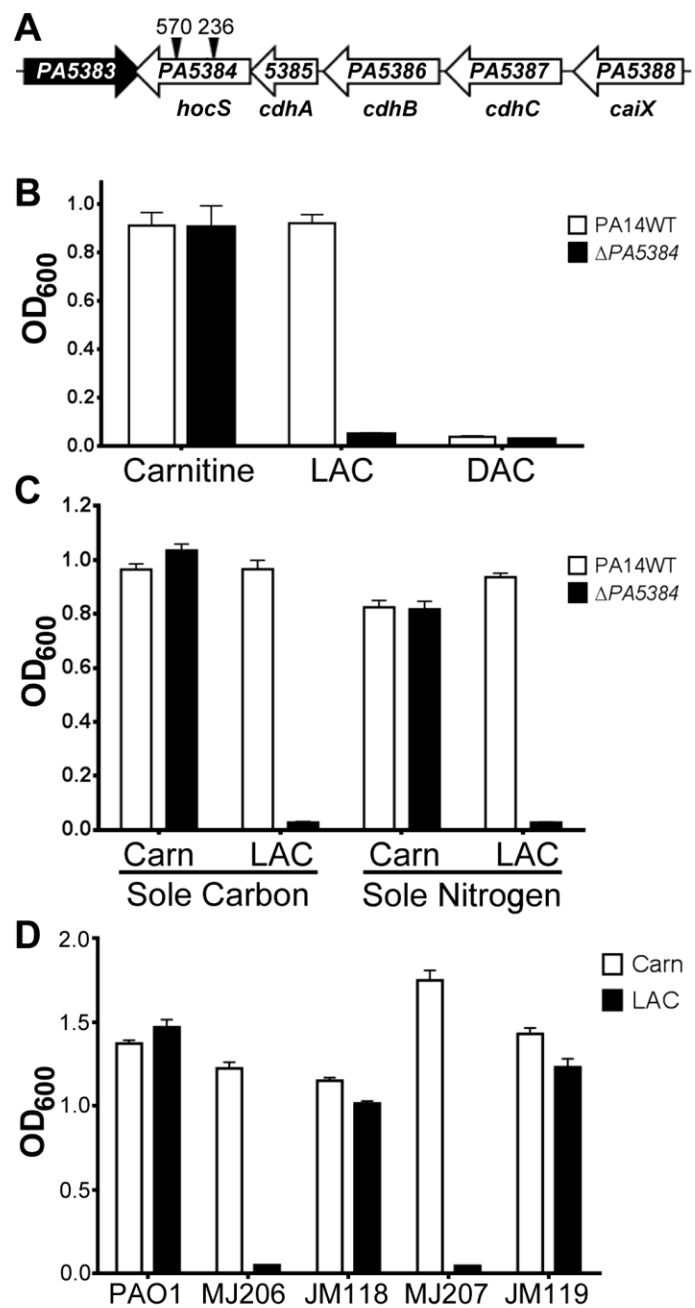
(A) Diagram of the carnitine catabolism operon in *P. aeruginosa* PAO1. ORFs are designated as arrows and are scaled relative to other ORFs. Black triangles represent transposon insertion sites in two separate PAO1 *PA5384* mutant strains with position of insertion noted by base pair number above the triangle. Gene designations are given below the arrows (19, 38) along with our proposed gene name for *PA5384*. (B) PA14 WT and  $\Delta PA5384$  mutant cells were grown in MOPS minimal media supplemented with 20mM of carnitine, L-acetylcarnitine (LAC), D-acetylcarnitine (DAC), or racemic mix of D- and L-acetylcarnitine (DLAC) as sole sources of carbon. (C) PA14 WT and  $\Delta PA5384$  mutant cells were grown in MOPS minimal media or MOPS minimal media without nitrogen supplemented with 20mM of carnitine (Carn) or L-acetylcarnitine (LAC) as the sole carbon and nitrogen source. (D) Trans complementation of two independent PAO1 *PA5384* transposon mutants. Cells were incubated in MOPS minimal media with 20 mM carnitine or LAC. MJ206 is the strain designation for the *PA5384::Tn* with transposon insertion at bp 570 and the derivative *attTn7::PA5384* strain is designated JM118. MJ207 is the strain designation for *PA5384::Tn* with transposon insertion at bp 236 and the derived *attTn7::PA5384* strain is designated JM119. Errors bars represent standard deviations of three replicates and results are representative of three independent experiments.

**Figure 2. PA5384 can hydrolyze L-acetylcarnitine.** Coupled enzymatic assay with purified 6xHis-PA5384 and substrates ( $\Delta$ ) 250  $\mu$ M L-acetylcarnitine (LAC), ( $\circ$ ) 125  $\mu$ M (LAC), ( $\blacklozenge$ ) 250  $\mu$ M D-acetylcarnitine (DAC), ( $\bullet$ ) 250  $\mu$ M D,L-acetylcarnitine (DLAC), ( $\blacksquare$ ) 250  $\mu$ M acetylcholine (AcCho), ( $\square$ ) no substrate, or ( $\blacktriangle$ ) 250  $\mu$ M LAC with no added PA5384. Lines have been added from the key to the absorbance curves to clarify the line identities. Errors bars represent standard deviations of triplicate samples and results are representative of three separate experiments.

**Figure 3. PA5384 encodes a short-chain acylcarnitine hydrolase.** (A) PA14 WT to 16C acylcarnitine chain lengths. Number on x-axis indicates chain length. The y-axis shows relative growth of the mutant compared to WT, expressed as a percent of WT and the  $\Delta$ PA5384 mutant were grown in MOPS minimal media with 20 mM of carnitine (0) or 2C growth (i.e. WT growth = 100%). Growth for carnitine to acylcarnitines with 12C chain lengths was measured by OD<sub>600</sub> and growth on acylcarnitines with chain lengths of 14C and 16C was determined by serial dilution and plating. (\*) Neither PA14 WT nor  $\Delta$ PA5384 mutant strain grew on octanoylcarnitine as the sole carbon source. (B) Free L-carnitine generated from hydrolysis of acylcarnitines by 6xHis-PA5384 was detected using a colorimetric L-carnitine assay. The dotted line represents the assay sensitivity limit, below which measurements of carnitine are not reliable. Errors bars represent standard deviations of triplicate samples and results are representative of three separate experiments.

**Figure 4. HocS is likely localized to the cytoplasm.** Cells from two independent strains, HocS-FLAG-1 (JM116) and HocS-FLAG-2 (JM117) that integrate a C-terminal FLAG-tag on HocS, and control strain JM141 were grown in carnitine. The extracellular (E), soluble (S), and insoluble fractions (I) were prepared as described in the methods section. After SDS-PAGE and blotting, the FLAG-tag was detected with a monoclonal anti-FLAG antibody and the cytoplasmic control, SadB, was detected with an anti-SadB antibody. The western blot shown is a representative of four comparable blots.

**Figure 5. HocS has a role in osmoprotection and induction of the virulence factor *plcH*.** (A) Osmoprotection was determined by growing PA14 WT and  $\Delta hocS$  mutant cells for 48 hours in MOPS minimal media (50 mM NaCl) supplemented with 800 mM NaCl, 20 mM glucose as the carbon source and with the compatible solutes carnitine or L-acetylcarnitine (LAC) at a final concentration of 0.2 mM. (B) Induction of *plcH-lacZYA* (pMW22) by carnitine (Carn) or LAC was measured by inducing PA14 WT and  $\Delta hocS$  mutant strain cells for 4 hours prior to the  $\beta$ -galactosidase assay. (C) To measure PLC activity, PA14 WT and  $\Delta hocS$  mutant cells were grown overnight in MOPS supplemented with 20 mM pyruvate and either 1 mM carnitine or LAC. The resultant culture supernatants were used in an NPPC assay to determine PLC activity. Error bars represent standard deviations of triplicate samples and results are representative of three separate experiments.



**Figure 1. *PA5384* is required for *P. aeruginosa* growth on L-acetylcarnitine.**

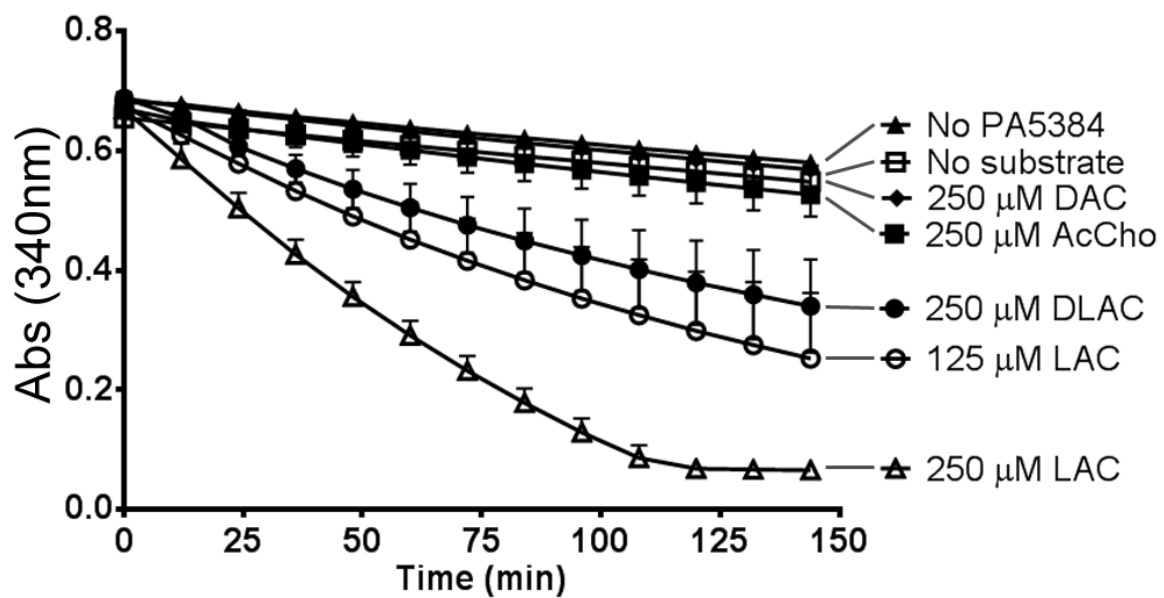


Figure 2. PA5384 can hydrolyze L-acetylcarnitine.



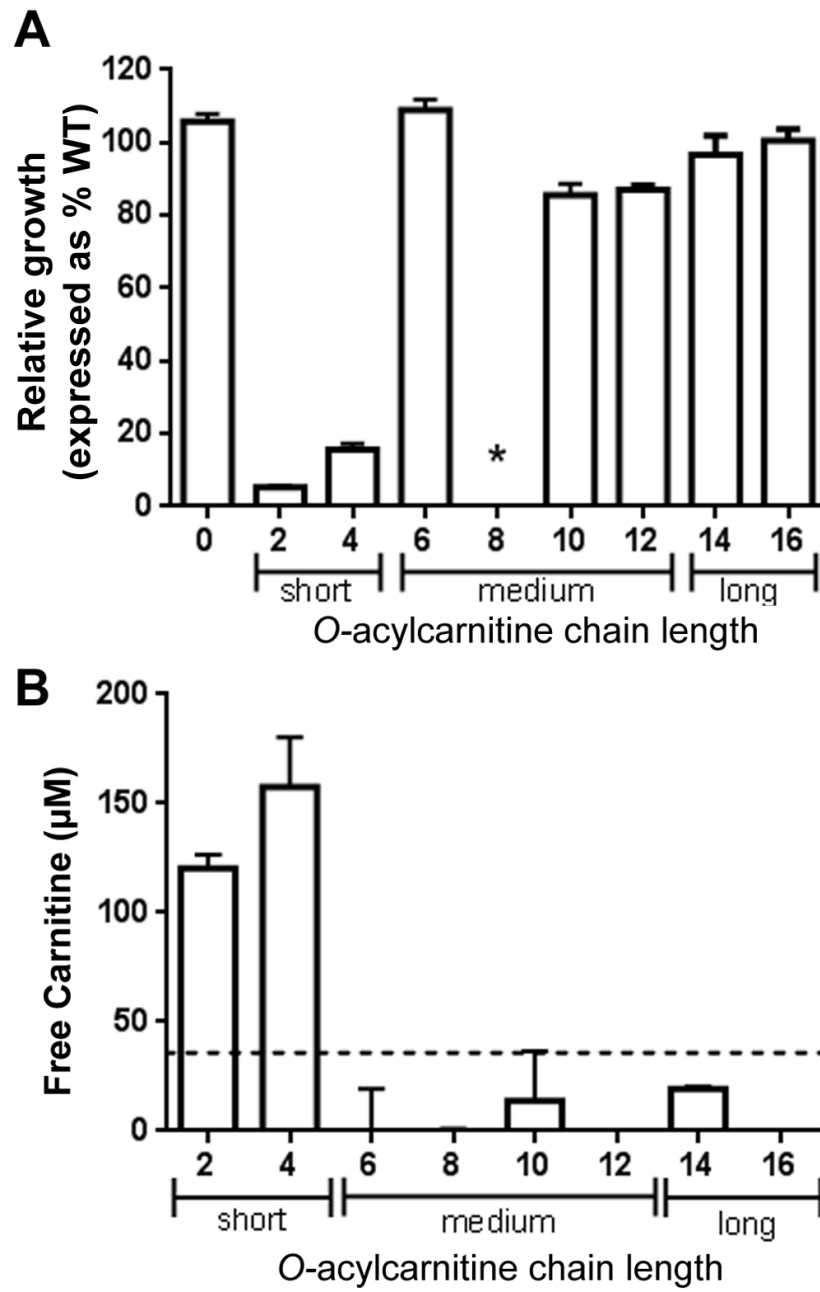
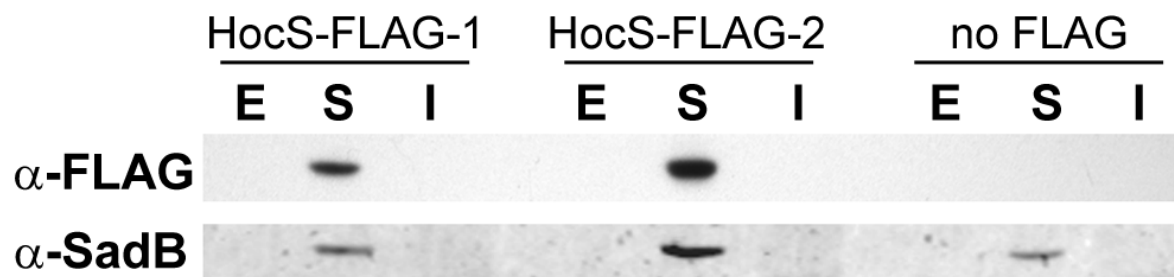
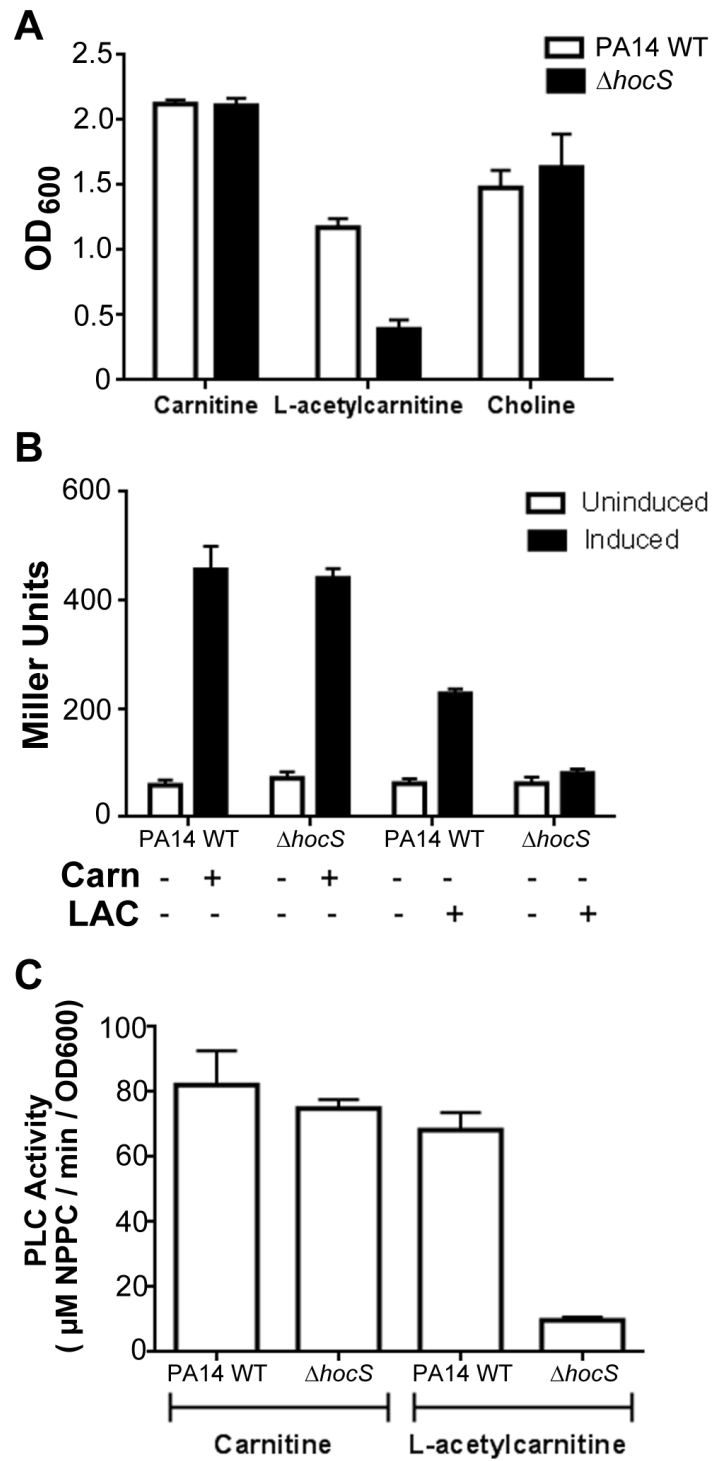


Figure 3. *PA5384* encodes a short-chain acylcarnitine hydrolase.



**Figure 4. HocS is likely localized to the cytoplasm.**



**Figure 5. HocS has a role in osmoprotection and induction of the virulence factor *plcH*.**

## 2.8. Tables

**Table 1. Strains and plasmids used in this study**

Strain or plasmid	Database no.	Description (reference or source) <sup>a</sup>
<i>P. aeruginosa</i> PAO1		
PAO1 wild type	MJ79	<i>P. aeruginosa</i> WT (25)
PA5384::Tn5	MJ206	PAO1 transposon mutant ID 47947, position in ORF 570 (31)
PA5384::Tn5	MJ207	PAO1 transposon mutant ID 52172, position in ORF 236 (31)
PA5384::Tn5 <i>att</i> Tn7::PA5384	JM118	<i>trans</i> -complementation of PA5384::Tn ID 47947 (this study)
PA5384::Tn5 <i>att</i> Tn7::PA5384	JM119	<i>trans</i> -complementation of PA5384::Tn ID 52172 (this study)
<i>P. aeruginosa</i> PA14		
PA14 wild type	MJ101	<i>P. aeruginosa</i> WT (24)
ΔPA5384	MJ264	Clean PA5384 deletion (this study)
PA5384-FLAG	JM116	PA14 with PA5384 C-terminal FLAG-tag clone 1 (this study)
PA5384-FLAG	JM117	PA14 with PA5384 C-terminal FLAG-tag clone 2 (this study)
PA14 pMQ30 integrant	JM141	PA14 with PA4921-pMQ30 genome integrant (this study)
<i>E. coli</i>		
<i>E. coli</i> wild type	MJ340	S17/λpir
<i>E. coli</i> T7 Express		NEB C2566
<i>E. coli</i> NEB5α		NEB C2987
<i>E. coli</i> T7 Express with pJAM8	JM17	T7 Express with pJAM8 (this study)
Plasmids		
pMQ30		Suicide vector, Gm <sup>r</sup> (27)
pMQ80		High-copy-number <i>Pseudomonas</i> vector, Gm <sup>r</sup> (27)
pET-30a		T7 expression vector, 6× His N-terminal tag, Kan <sup>r</sup> (Novagen)
pUCP22		High-copy-number <i>Pseudomonas</i> stabilization vector, Gm <sup>r</sup> (49)
pUC18-mini-Tn7T-Gm		Gm <sup>r</sup> on mini-Tn7T (32)
pTNS2		Plasmid carrying the <i>att</i> Tn7 transposase (33)
pMW22		Promoter <i>plcH-lacZYA</i> transcriptional fusion (35)
pMW79		PA14 genomic clone with PA5380–PA5389 in pMQ8 (19)
pMW86		Causes deletion of PA5384 in pMQ30 (this study)
pJAM6		PA5384-PA5388 in pUCP22, Gm <sup>r</sup> (derived from pMW79) (this study)
pJAM8		PA5384 in pET-30a expression vector (this study)
pJAM10		PA14 PA5384 in pMQ30 (this study)
pJAM34		PA14 PA5384FLAG, C terminal (this study)
pJAM61		PA14 PA5388 promoter in pJAM8 (this study)
pJAM62		PA14 PA5388 promoter fused to PA5384 in pUC18-mini-Tn7T-Gm (32 and this study)

<sup>a</sup> ORF, open reading frame.

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## **CHAPTER 3:**

### **THE ROLE OF *cbcWV* AND *caiX* IN THE TRANSPORT OF *O*-ACYLCARNITINES IN *PSEUDOMONAS AERUGINOSA***

### 3.1. Abstract

The opportunistic pathogen *P. aeruginosa* can metabolize the compounds carnitine and acylcarnitines, which are abundant in host muscle and cardiac tissues. Acylcarnitines can be degraded to carnitine, where the liberated carnitine and its catabolic product glycine betaine can be used as osmoprotectants, to induce the virulence factor phospholipase C, and can function as sole carbon, nitrogen, and energy sources. *P. aeruginosa* is incapable of *de novo* synthesis of carnitine and acylcarnitines and they therefore must be imported from exogenous source. In this study we present the first characterization of acylcarnitine transport. Short-chain acylcarnitines are imported by the ABC transporter CaiX and CbcWV. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically and the free carnitine is transported through CaiX-CbcWV.

### 3.2. Introduction

Carnitine and acylcarnitines are quaternary amine compounds (QAC) that are present in all domains of life and are found in soil [1, 2] and plants [3, 4], and are abundant in animal tissues [5, 6]. The majority of carnitine is in its free form, but a portion is acylated, with acetylcarnitine being the most abundant acylcarnitine [7]. In animals, fatty acids are brought into the mitochondria via the carnitine shuttle where they undergo beta oxidation to produce energy for the cell. There are no animal enzymes capable of degrading carnitine, therefore any breakdown is from bacteria [8].

*Pseudomonas aeruginosa* is a Gram-negative bacterium that is nearly ubiquitous in nature and is an opportunistic pathogen that is capable of metabolizing carnitine [9]

and acylcarnitines [10]. Short-chain acylcarnitines are hydrolyzed in the cytosol by HocS [10], but the medium- and long-chain acylcarnitine hydrolase(s) remains unidentified. Acylcarnitine hydrolysis yields a free fatty acid and carnitine. The resulting free carnitine and its catabolic product glycine betaine function as osmoprotectants in hyperosmotic environments [10-12], induce the virulence factor phospholipase C, *plcH* [11], and can be used as sole carbon, nitrogen, and energy sources [10, 13, 14]. The liberated fatty acid can also be used as a sole carbon source [15] and the *faoAB* (*fadBA5*) genes responsible for oxidation of fatty acids twelve carbons and longer were found to be strongly induced in cystic fibrosis sputum [16] and important for *P. aeruginosa* fitness in mouse wound models [17].

To date, de novo synthesis of carnitine has not been demonstrated in bacteria [18], therefore it must be acquired from the environment. There are two routes to carnitine transport: first, a BCCT (betaine/choline/carnitine) transporter, driven by sodium motive force, proton motive force, or substrate:product antiport [19]; and second, an ATP-binding cassette (ABC) transporter powered by ATP hydrolysis. In bacteria, ABC transporters are comprised of multiple proteins: a transmembrane domain, a nucleotide binding domain (ATPase), and a substrate binding protein (periplasmic binding protein) [20]. In *P. aeruginosa*, the genes encoding the ABC transporter for carnitine import are located in two different operons. The genes encoding the transmembrane domain (*cbcW*) and the nucleotide binding domain (*cbcV*) are located in the *cbcXWV* operon (*PA5378-PA5376*) [21], while the *caiX* gene, encoding the carnitine substrate binding protein is located in the carnitine catabolism operon, *caiXcdhCABhocS*

(PA5388-PA5384) [22]. In *P. aeruginosa* CbcWV interacts with three different substrate binding proteins, CaiX, BetX, and CbcX, all of which bind ligand that are QACs: carnitine, glycine betaine, and choline (Fig. 1) [21]. *caiX* and *cbcWV* were previously shown to be required for growth on carnitine [21, 22], but the mechanism for acylcarnitine import and utilization in *P. aeruginosa* had not been studied.

Here we report the route of acylcarnitine transport in *P. aeruginosa* (Fig. 7). Through growth and transcriptional reporter assays, we demonstrate that medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically by an unknown hydrolase(s) to yield carnitine and a fatty acid. Carnitine and short-chain acylcarnitines are imported by the ABC transporter CbcWV and the substrate binding protein CaiX. *cbcWV* is only necessary for utilization of medium- and long-chain acylcarnitines as sole nitrogen sources but not osmoprotectants. *cbcWV* is necessary for short-chain acylcarnitines to function as osmoprotectants, but carnitine, medium-, and long-chain acylcarnitines are imported by an undefined transporter to provide osmoprotection.

### 3.3 Materials and Methods

#### 3.3.1. Strain, growth conditions, and compounds

*P. aeruginosa* PA14 wild type [23],  $\Delta caiX$  [22], and  $\Delta cbcV$  [24] strains were maintained on PIA plates or in LB liquid and when necessary with 50  $\mu\text{g ml}^{-1}$  or 40  $\mu\text{g ml}^{-1}$  of gentamicin, respectively. Carnitine and acylcarnitines were purchased from the following vendors: L-carnitine (Acros); L-acetylcarnitine and DL-palmitoylcarnitine

(Sigma); L-butyrylcarnitine (Crystal Chem Inc); and DL-hexanoyl-, DL-octanoyl-, DL-decanoyl-, DL-lauroyl-, and DL-myristoylcarnitine (BioTrends).

### **3.3.2. Growth assays**

To test the growth of strains carrying deletions in transport components on carnitine and acylcarnitines (2-6 and 10-16 fatty acid chain length) as sole carbon sources, PA14 wild type,  $\Delta caiX$ , or  $\Delta cbcV$  were grown overnight at 37 °C in minimal media MOPS with 20 mM pyruvate and 5 mM glucose. Cells were then collected by centrifugation, washed, and inoculated at a final OD<sub>600</sub> nm of 0.05 in MOPS with 20 mM of the individual acylcarnitine or carnitine. Cultures were grown at 37 °C in a 48-well plate on a horizontal shaker for 24 hours. OD<sub>600</sub> was measured at the end of the assay on a BioTek plate reader. Due to insolubility, growth on palmitoylcarnitine growth was determined by CFU counts.

To test the use of carnitine and acylcarnitines as a sole nitrogen source, overnight cultures and preparation of the cells were the same as above. Cells were inoculated into minimal media M63 nitrogen free containing 20 mM pyruvate and 2 mM of the acylcarnitine. Palmitoylcarnitine samples had 0.75% deoxycholate added to prevent precipitation. Cultures were grown at 37 °C in a 48-well plate on a plate shaker for 24 hours. All acylcarnitine growth assays were measured on the BioTek plate reader at OD<sub>600</sub>.

### 3.3.3. Transcriptional reporter assay

To assess induction of *caiX* in response to acylcarnitines in the transport related deletion strains  $\Delta caiX$  and  $\Delta cbcV$ , we used the transcriptional reporter *PcaiX-lacZYA* (pJAM22; construction described in Chapter 4). PA14WT,  $\Delta caiX$ , or  $\Delta cbcV$  carrying pJAM22 were grown overnight at 37 °C in MOPS medium, 20 mM pyruvate, 5 mM glucose, and 20  $\mu\text{g ml}^{-1}$  gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS. Cells were inoculated at a final OD<sub>600</sub> of 0.05 into a 48-well plate containing MOPS medium, 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and 1 mM of the induction compound (carnitine or acylcarnitines of 2-16 fatty acid chain length). Cells were incubated for 4 hours at 37 °C while shaking and then a  $\beta$ -galactosidase assay was performed according to Miller [25]. Due to insolubility of palmitoylcarnitine, OD<sub>600</sub> for those wells was measured prior to inoculation with cells and subtracted from the final OD<sub>600</sub> nm reading.

### 3.3.4. Osmoprotection assay

Osmoprotection assays were done as previously described with a few modifications [10]. Briefly, *P. aeruginosa* PA14 wild type and  $\Delta cbcV$  were grown overnight in MOPS medium supplemented with 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended to an OD<sub>600</sub> of 0.05 in MOPS medium with an additional 800 mM NaCl (850 mM total NaCl), 20 mM glucose, and 0.2 mM osmoprotectant (carnitine, acetylcarnitine, decanoylcarnitine, or

myristoylcarnitine). 48-well plates were shaken at 37 °C for 48 hours and growth was determined by OD<sub>600</sub>.

### 3.4. Results

#### 3.4.1. *cbcV* is required for growth and induction of *caiX* in response to short-chain acylcarnitines.

In previous studies we showed that *P. aeruginosa* can grow on the short-chain acylcarnitines (SCAC) acetyl- and butyrylcarnitine [10]. The SCACs are hydrolyzed in the cytoplasm by HocS, therefore they must be imported to be used as sole carbon and nitrogen sources. It has been shown that the substrate binding protein *caiX* [22] and the core components of the ABC transporter *cbcWV* are required for growth on carnitine [21] (Fig. 2A). We hypothesized that this ABC carnitine transport system has broader specificity and can import short-chain acylcarnitines as well. The *cbcV* deletion strain was not able to grow on SCAC as the sole carbon source, (Fig. 2). Based on the growth data and HocS's cytoplasmic location, we predicted that *cbcWV* was necessary for SCAC induction of *caiX*, the first gene in the carnitine catabolism operon (Fig. 1). PA14WT and *cbcV* deletion mutant carrying the *PcaiX-lacZ* plasmid (pJAM22) were induced with carnitine and SCACs, and *caiX* transcription was assessed by  $\beta$ -galactosidase assays. Carnitine and SCACs induced transcription of *caiX* in wild type but in the *cbcV* deletion mutant induction was greatly reduced due to the lack of the compounds not entering the cytoplasm (Fig. 2B). Taken together, the growth and reporter data indicate *cbcWV* is required for transport of SCACs.

### 3.4.2. CaiX is the substrate binding protein for short-chain acylcarnitines.

CbcWV is the core ABC transporter for three QAC substrate binding proteins (SBP) – CaiX, BetX, and CbcX [21]. Since *cbcWV* is required for transport of SCACs (Fig. 2), this led us to investigate if CaiX was the SBP for SCACs, which the alternate possibility that there may be a separate SBP that interacts with CbcWV for SCAC transport. To determine if *caiX* is required for SCAC transport we compared *P. aeruginosa* PA14 wild type to the *caiX* mutant for their ability to grow on SCACs as sole nitrogen sources and  $\beta$ -galactosidase assays were performed as above on PA14 wild type and *caiX* carrying the *PcaiX-lacZ* plasmid. The *caiX* mutant was unable to grow on SCACs (Fig. 3A) and induce the carnitine operon (Fig. 3B). These results support two conclusions, (i), *caiX* is required for growth on SCACs, and (ii), the ligand specificity of CaiX can be expanded to include acetyl- and butyrylcarnitine.

### 3.4.3. Growth on medium- and long-chain acylcarnitines does not require *cbcV*.

Animal and plant cells use medium- and long chain acylcarnitines as sources for and intermediate in beta-oxidation [26, 27], and we previously demonstrated that *P. aeruginosa* can use MCAC and LCAC as sole carbon sources [10]. We next tested if *cbcWV* was required for growth on MCAC and LCAC as sole carbon sources. PA14 wild type and the *cbcV* deletion mutant strain were able to grow in MOPS minimal media with acylcarnitines 6C and 10C-16C fatty acid chain length as the sole carbon source (Fig. 4). These data suggest that MCAC and LCAC require an entirely different transport system



or are hydrolyzed in the outside of the cytosol where the free fatty acid can serve as a carbon source.

#### **3.4.4. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically.**

To determine the route of medium- and long-chain acylcarnitine being transport, we used the carnitine responsive transcriptional reporter *P<sub>caiX</sub>-lacZ<sub>YA</sub>*. [22]. This rationale is that carnitine in the cytosol leads to *caiX* induction. If acylcarnitines can be transported into the cytoplasm and subsequently hydrolyzed, the free carnitine will induce *caiX*, whereas hydrolysis outside the cytosol would require the liberated carnitine to be transported via *CaiX* and *CbcWV*. Therefore, dependence on the free carnitine transporter components can allow us to identify the location of medium- and long-chain hydrolysis. Here we show that the induction of *caiX* is decreased by over three fold in a *caiX* and *cbcV* deletion mutant compared to the wild type for 6C-16C acylcarnitines, showing that *caiX* and *cbcV* are needed for the induction of *caiX* (Fig. 5A-B). The residual induction in these mutants will be addressed in the discussion section.

The lack of induction of *caiX* while retaining the ability to grow in a *cbcV* mutant when MCAC and LCAC were the sole carbon sources, led us to postulate that hydrolysis of these compounds occurs outside of the cytosol. A growth assay using 6C-16C acylcarnitines as sole nitrogen sources revealed that the *caiX* and *cbcV* deletion mutants failed to grow when compared to wild type (Fig. 5C). Along with the carbon source growth data and induction of *caiX* (Fig 5A-B, Fig. 4) indicates that hydrolysis of MCAC

and LCAC occurs extracytoplasmically by an unidentified hydrolase and CaiX binds the liberated carnitine for transport through CbcWV as discussed in detail in the discussion.

#### **3.4.5. Osmoprotection by short-chain acylcarnitines requires *cbcWV*.**

It has been demonstrated that *cbcWV* is important for growth on choline but is not necessary for osmoprotection in a hyperosmotic environment when choline is the osmoprotectant [24]. We predict this is similar for carnitine and that another transporter is sufficient for osmoprotection. PA14 wild type and the *cbcV* mutant were grown in MOPS media with a final NaCl concentration of 850 mM, 20 mM of glucose as the carbon source, and 0.2 mM of the osmoprotectants carnitine, acetylcarnitine, decanoylcarnitine, or myristoylcarnitine. Carnitine, decanoylcarnitine, and myristoylcarnitine can be osmoprotectants in a *cbcV* mutant whereas acetylcarnitine cannot (Fig. 6). Carnitine and the free carnitine generated from hydrolysis outside the cytoplasm can circumvent *cbcV* by being imported through an unknown transporter to achieve osmoprotection. Acetylcarnitine, on the other hand, must first be imported into the cytoplasm by CbcWV for HocS hydrolysis in the cytosol in order to serve as an osmoprotectant [10].

### **3.5. Discussion**

Acylcarnitines are valuable carbon and nitrogen sources, and its catabolic products carnitine and GB can function as osmoprotectants and induces the virulence factor phospholipase C, *plcH* [10]. In *P. aeruginosa* carnitine is transported by the substrate binding protein CaiX and the ABC transporter CbcWV but no work to date has

examined acylcarnitine import [21, 22]. Our goal was to understand how acylcarnitines are imported and determine if there is any functional overlap between carnitine and acylcarnitine transport system. Using growth and reporter assays we show that MCAC and LCAC are hydrolyzed extracytoplasmically and the free carnitine is subsequently imported by CaiX and CbcWV to be utilized as a sole nitrogen source. Carnitine and the free carnitine generated from MCAC and LCAC hydrolysis can function as an osmoprotectant, but *cbcWV* is not required. SCAC require *caiX* and *cbcWV* for transport into the cytoplasm and are thus necessary for osmoprotection, expression of *plcH*, and use as carbon and nitrogen sources.

OpuC is an ABC transporter in *Bacillus subtilis* that imports the structurally similar QAC compounds carnitine, crotonobetaine, and gamma butyrobetaine [28]. The *P. aeruginosa* ABC transporter CbcWV is similar, and imports multiple QACs: glycine betaine, choline, and carnitine [21]. This led us to inquire if *cbcWV* imports other QACs and is involved in acylcarnitine import. Using growth assays with 2C-16C acylcarnitines (excluding octanoylcarnitine) as sole carbon sources, the *cbcV* mutant only had a defect on SCACs indicating that *cbcV* is not required for growth of MCAC and LCACs (Figs. 2 and 4). The substrate binding protein is the other essential component of an ABC transporter. Not all substrate binding proteins have a single ligand and some can promiscuously bind structurally similar compounds, as seen in *B. subtilis* [28, 29], *P. aeruginosa* [21], *E. coli* [30], and others [31]. In this study we have extended the binding specificity of CaiX to encompass acetyl- and butyrylcarnitine, as a *caiX* deletion mutant fails to grow on SCACs as sole carbon or nitrogen source. These data suggested two

possible methods of transport of MCAC and LCACs: extracytoplasmic hydrolysis yielding carnitine and a fatty acid, or alternatively, import is through an unidentified transporter and cytoplasmic hydrolysis, in a manner similar to the SCACs [10].

Kappes and Bremer showed that *Bacillus subtilis* can hydrolyze acetyl- and octanoylcarnitine but were not able to determine if hydrolysis was on the cell surface or inside the cell [28]. Using the *PcaiX-lacZ* reporter and nitrogen growth assays, we showed that MCAC and LCACs are hydrolyzed outside the cytoplasm and the liberated carnitine is transported through CbcWV (Figs. 4 and 5). The ability of the *cbcV* and *caiX* mutants to use MCAC and LCACs as sole carbon sources but not as sole nitrogen sources indicates that the fatty acid is the predominant carbon source in this compound (Fig. 5). In the *caiX* and *cbcV* mutants there is residual *PcaiX-lacZ* induction in the carnitine and acylcarnitine samples, which we speculate is due to transport of carnitine through an unidentified BCCT transporter.

We previously reported that *P. aeruginosa* cannot utilize octanoylcarnitine as a sole carbon source [10]. Here we show octanoylcarnitine minimally induces *caiX* compared to carnitine in wild type *P. aeruginosa* (Fig. 5), indicating that hydrolysis is inefficient and is likely responsible for the inability of *P. aeruginosa* to grow on octanoylcarnitine [10]. Caprylic acid, the 8C chain fatty acid produced from octanoylcarnitine hydrolysis, has antibacterial properties and is commonly used in food preservation, medicine, cosmetics, and agriculture [32]. Caprylic acid and other fatty acids inhibit growth or kill bacteria, such as *Salmonella* Enteritidis [33], *E. coli* O157:H7 [34], *S. aureus* [35], and *P. aeruginosa* [35-37], by disrupting the cell membrane and

interfering with processes associated within or at the membrane such as electron transport, oxidative phosphorylation [32]. The outer membrane acts as a semi-permeable barrier and defense mechanism [32, 38], but octanoylcarnitine may circumvent this natural barrier if hydrolysis is periplasmic, producing caprylic acid in the periplasm. *P. aeruginosa* may have evolved a hydrolase that excludes octanoylcarnitine as a substrate, in part to evade the antimicrobial effects of caprylic acid.

Acylcarnitine and carnitine transport into the cell for osmoprotection is well established [18]. Glycine betaine is the catabolic product of both carnitine and choline catabolism. Initial import of choline is through BetT1 and BetT3, with BetT3 having additional roles in growth and osmoprotection [24]. Choline is catabolized to glycine betaine which then induces *cbcWV* in a GbdR dependent manner, making *cbcWV* important for growth but not osmoprotection [24, 39]. Carnitine osmoprotection seems to function similarly, with *cbcWV* being required for growth but not osmoprotection (Fig. 6). A carnitine transporter sufficient for osmoprotection has not been identified but may be a BCCT transporter, as with choline, and may even be one of the BetT family of transporters.

This study expands our knowledge on *P. aeruginosa* metabolism and transport of acylcarnitines. MCAC and LCACs are hydrolyzed extracytoplasmically by an unknown hydrolase to generate carnitine and a free fatty acid. Although *cbcWV* is not necessary for MC- and LCACs to be used as carbon sources, *CbcWV* provides a route for liberated carnitine to be used as a nitrogen source. The liberated carnitine can be used as an osmoprotectant via transport through an identified transporter likely a BCCT. We were

also able to expand the ligand specificity of CaiX to include acetyl-, and butyrylcarnitine which are imported via the ABC transporter CbcWV, which is required for SCAC use as osmoprotectants and as carbon and nitrogen sources.

### 3.6. Figures

#### **Figure 1. Diagram of the operon configuration and ABC transporter in *P.***

*aeruginosa* PA14 [21]. (A) Diagram of the *P. aeruginosa* PAO1 choline ABC transporter operon *cbcXWV*: choline substrate binding protein, *cbcX*; transmembrane protein, *cbcW*; and nucleotide binding protein, *cbcV*. (B) Diagram of the carnitine catabolism operon *caiXcdhCABhocS*: carnitine substrate binding protein, *caiX*; beta-keto cleavage acid enzyme, *cdhC*; carnitine dehydrogenase protein, *cdhAB*; and short-chain *O*-acylcarnitine hydrolase, *hocS*.

#### **Figure 2. *cbcV* is necessary for growth and induction of *caiX* on short-chain**

**acylcarnitines.** (A) PA14WT and  $\Delta cbcV$  cells were grown in MOPS minimal media supplemented with 20 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cultures were grown at 37 °C for 24 hours prior to reading them at OD600 nm. (B) PA14 WT and  $\Delta cbcV$  carrying *caiXlacZ* plasmid (pJAM22) were grown in MOPS, 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and with or without 1 mM inducing compound carnitine, acetylcarnitine or butyrylcarnitine. Cells were induced for 4 hours at 37 °C before performing a Miller assay. AC is acetylcarnitine and BC is butyrylcarnitine. Two way ANOVA. ns, not significant; \*\*\*\*,  $P < 0.0001$ .

#### **Figure 3. *CaiX* is the substrate binding protein for short-chain acylcarnitines.** (A)

PA14WT and  $\Delta caiX$  cells were grown in MOPS minimal media supplemented with 20 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cultures were grown at 37 °C for 24

hours prior to reading them at OD600 nm. (B) PA14 WT and  $\Delta caiX$  carrying *caiXlacZ* plasmid (pJAM22) were grown in MOPS, 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and with or without 1 mM inducing compound carnitine, acetylcarnitine, or butyrylcarnitine. Cells were induced for 4 hours at 37 °C before performing a Miller assay. AC is acetylcarnitine and BC is butyrylcarnitine. Two way ANOVA. ns, not significant; \*\*\*\*,  $P < 0.0001$

**Figure 4. Medium- and long-chain acylcarnitines do not require *cbcV* for growth.**

PA14WT and *cbcV* deletion mutant were grown in MOPS with 20 mM of carnitine or acylcarnitines 6C, 10C-16C fatty acid chain length for 24 hours at 37 °C. OD600 nm was taken on a BioTek plate reader except for palmitoylcarnitine, which was determined by CFU due to micelle formation. One way ANOVA. ns, not significant; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$

**Figure 5. *caiX* and *cbcV* are required for growth when acylcarnitines are the sole nitrogen source.** (A) PA14WT (white bars),  $\Delta cbcV$  (gray bars), and  $\Delta caiX$  (black bars)

cells were grown at 37 °C in M63 minimal media supplemented with 20 mM pyruvate and 2 mM nitrogen source; ammonium, carnitine, or acylcarnitines 6C-16C fatty acid chain-length. Growth was assessed after 24 hours by OD600 nm. Two way ANOVA comparing carnitine to  $\Delta cbcV$  and  $\Delta caiX$ . ns, not significant; \*\*\*\*,  $P < 0.0001$  (B)

PA14WT was induced for 4 hours at 37 °C in MOPS with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and 1 mM carnitine, or 6C-16C acylcarnitines. Miller assays were

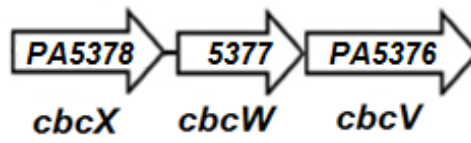


performed. ns, not significant; \*\*,  $P < 0.01$  (C) PA14WT,  $\Delta cbcV$ , and  $\Delta caiX$  were induced for 4 hours at 37 °C in MOPS with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and 1 mM carnitine, or 6C-16C acylcarnitines. Miller assays were performed. ns, not significant; \*\*\*\*,  $P < 0.0001$

**Figure 6. Short-chain acylcarnitines require *cbcWV* for osmoprotection in hyperosmotic conditions.** *P. aeruginosa* PA14WT and  $\Delta cbcV$  were grown in MOPS supplemented with 800 mM NaCl, 20 mM glucose, and with or without 0.2 mM osmoprotectants carnitine, acetylcarnitine, decanoylcarnitine, or myristoylcarnitine for 48 hours before reading the OD600 nm. ns, not significant; \*\*\*\*,  $P < 0.0001$

**Figure 7. Model of acylcarnitine import in *P. aeruginosa* PA14.**

A.

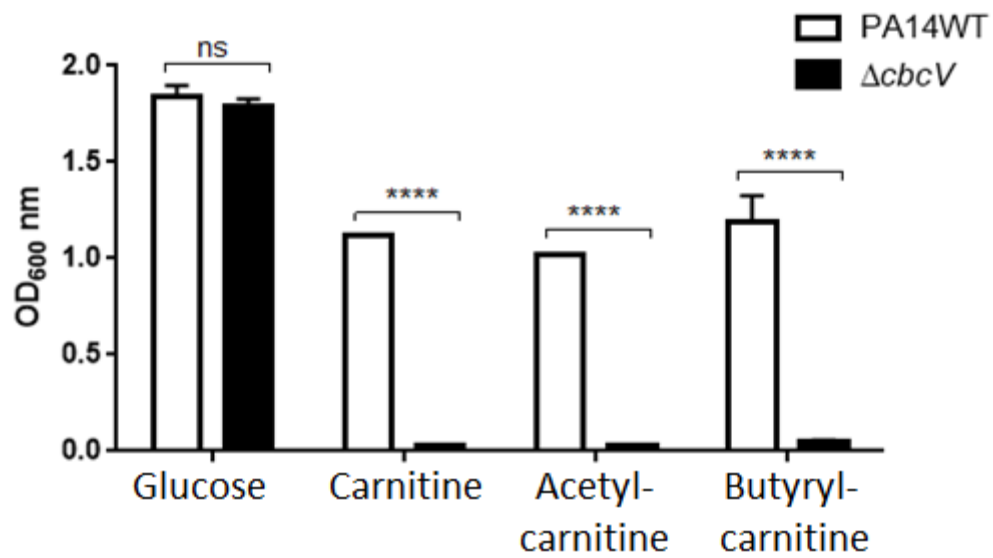


B.

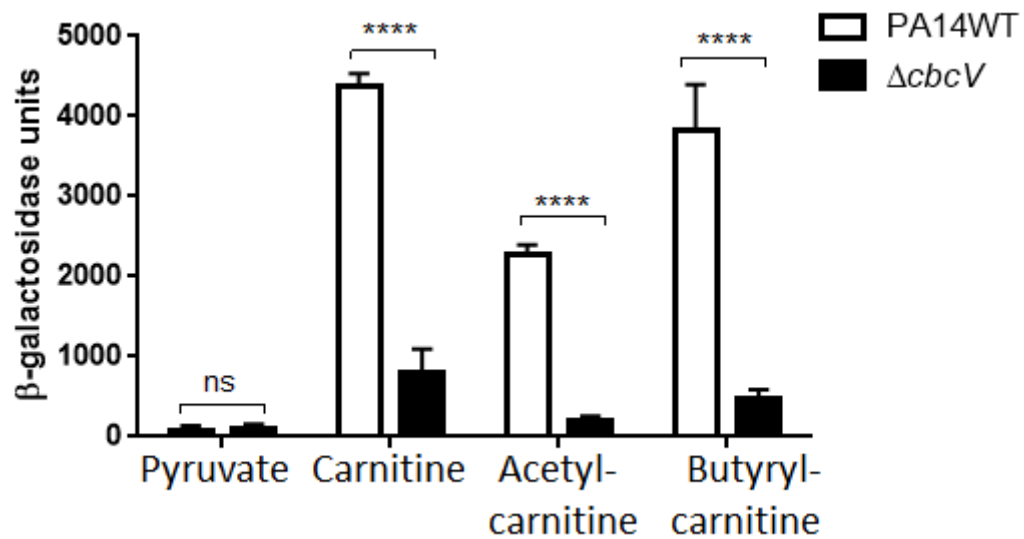


**Figure 1. Diagram of the operon configuration and ABC transporter in *P. aeruginosa* PA14.**

A.



B.



**Figure 2. *cbcV* is necessary for growth and induction of *caiX* on short-chain acylcarnitines.**

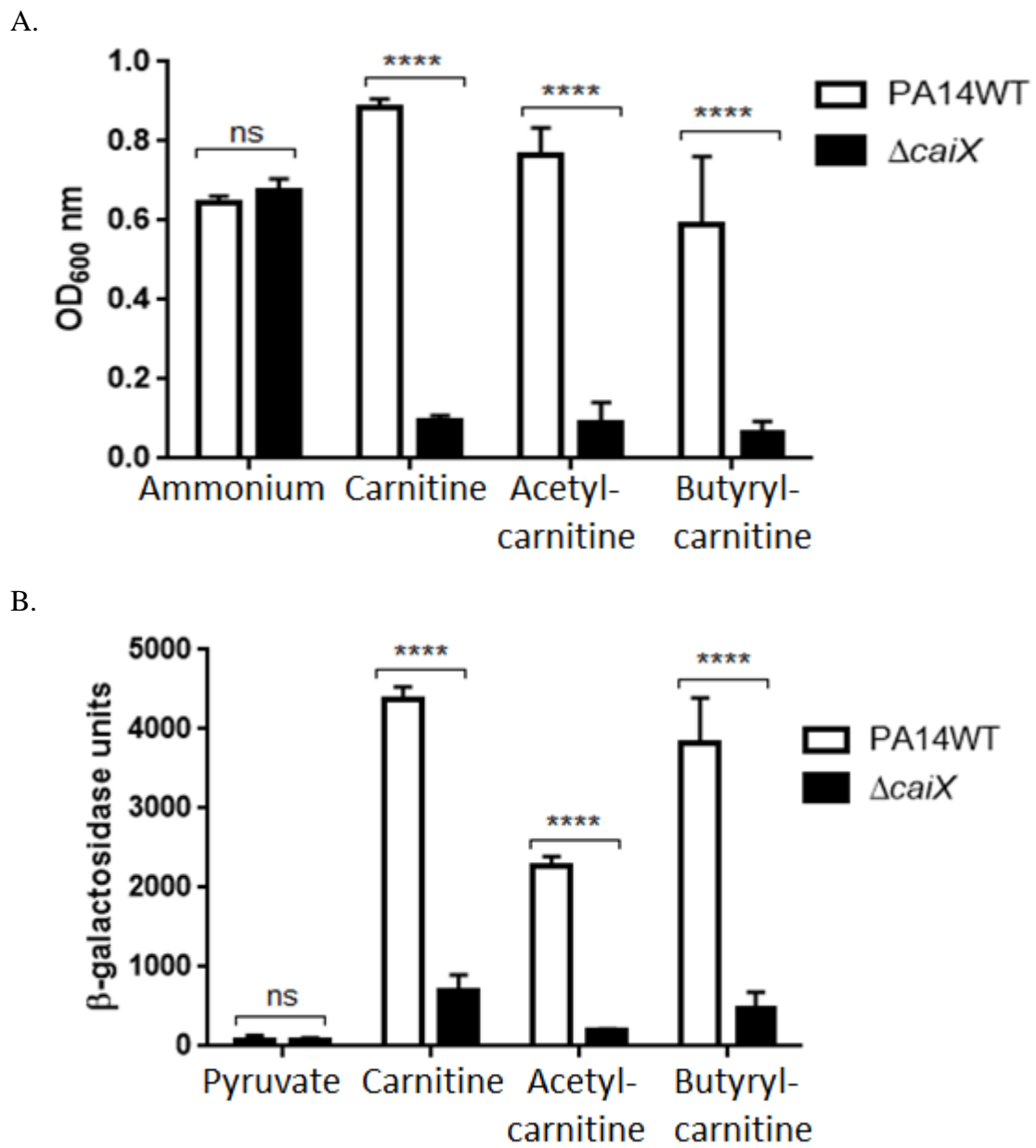


Figure 3. CaiX is the substrate binding protein for short-chain acylcarnitines.

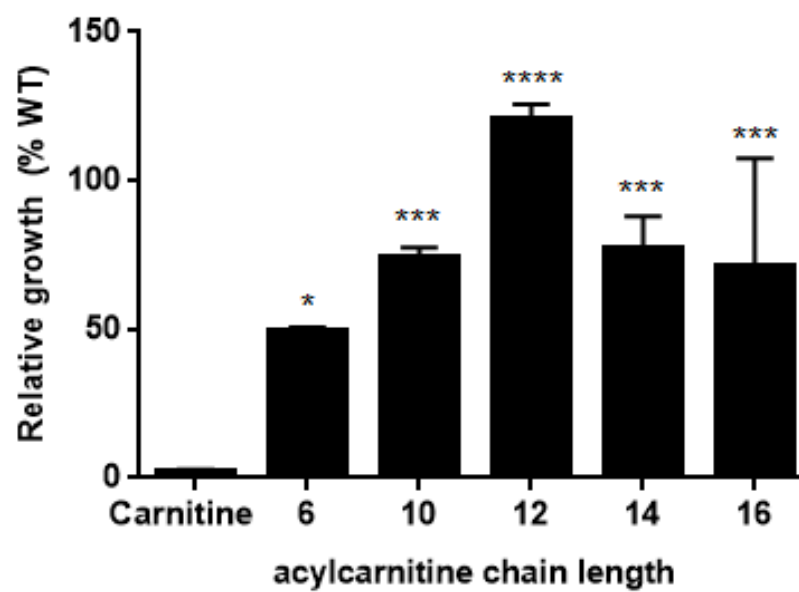
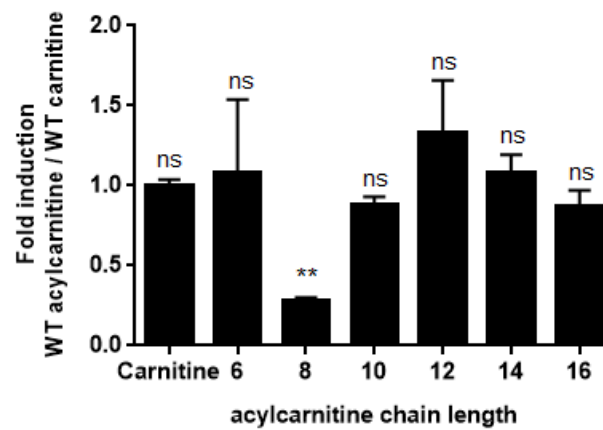
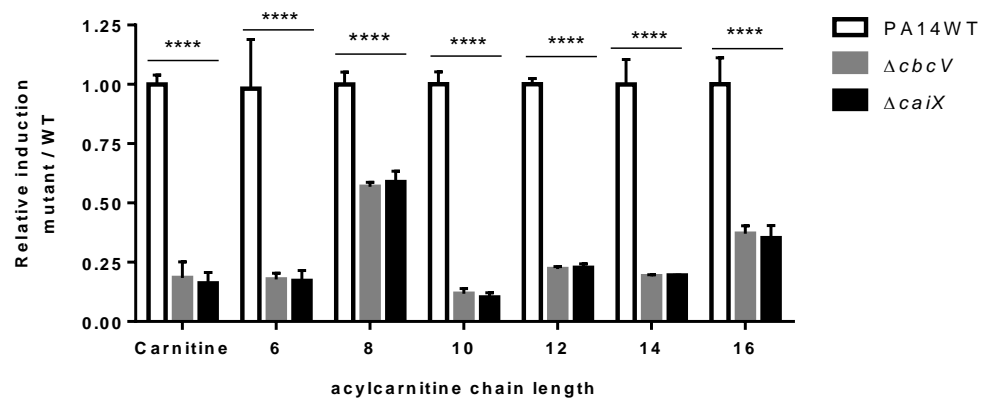


Figure 4. Medium- and long-chain acylcarnitines do not require *cbcV* for growth.

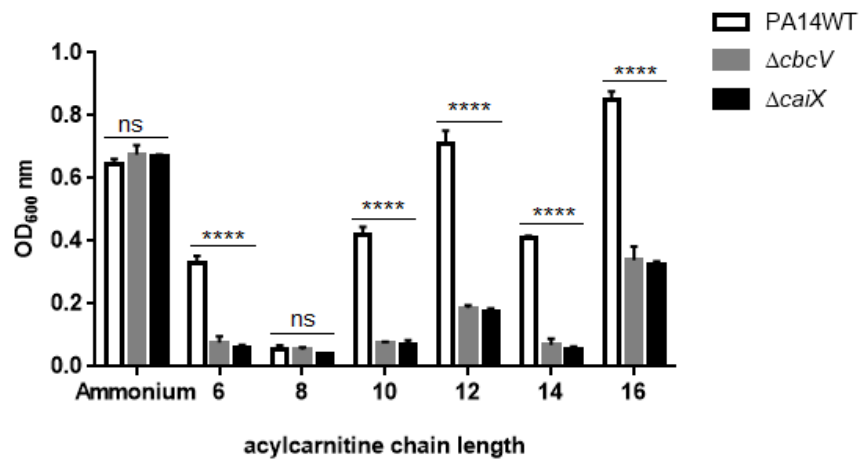
A.



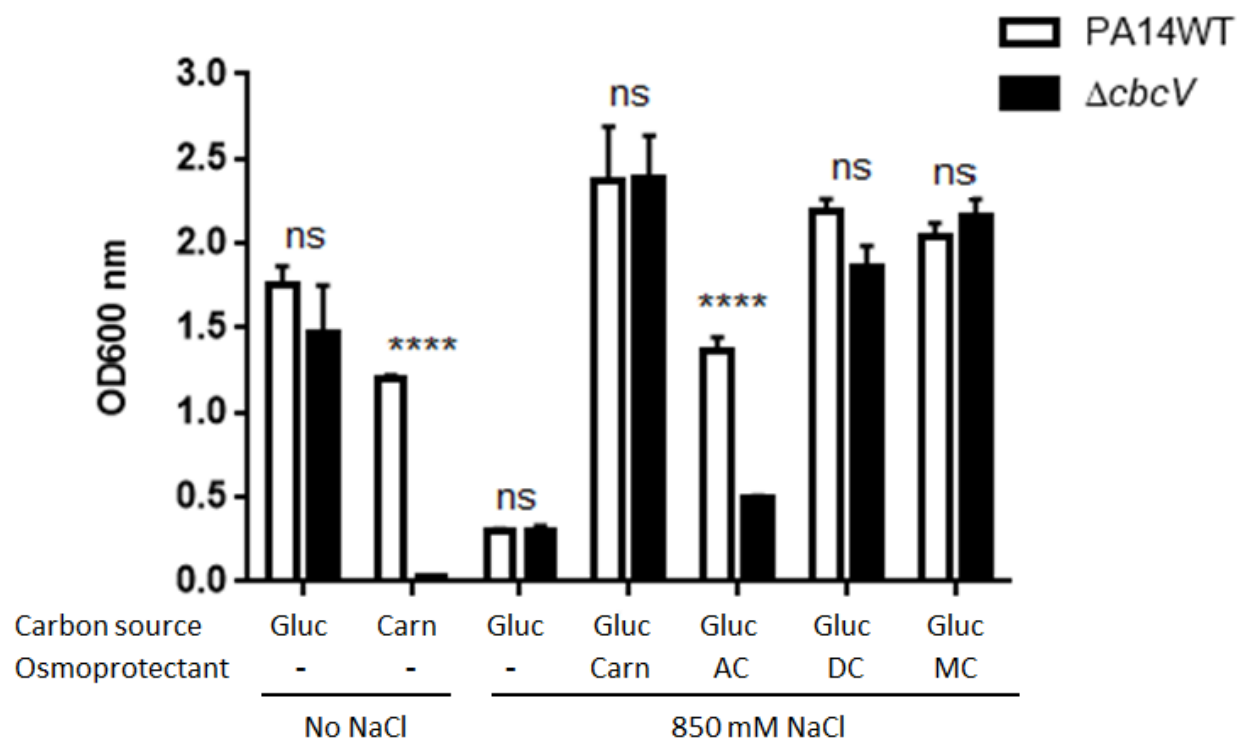
B.



C.



**Figure 5. *caiX* and *cbcV* are required for growth when acylcarnitines are the sole nitrogen source.**



**Figure 6. Short-chain acylcarnitines require *cbcWV* to function as osmoprotectants in hyperosmotic conditions.**

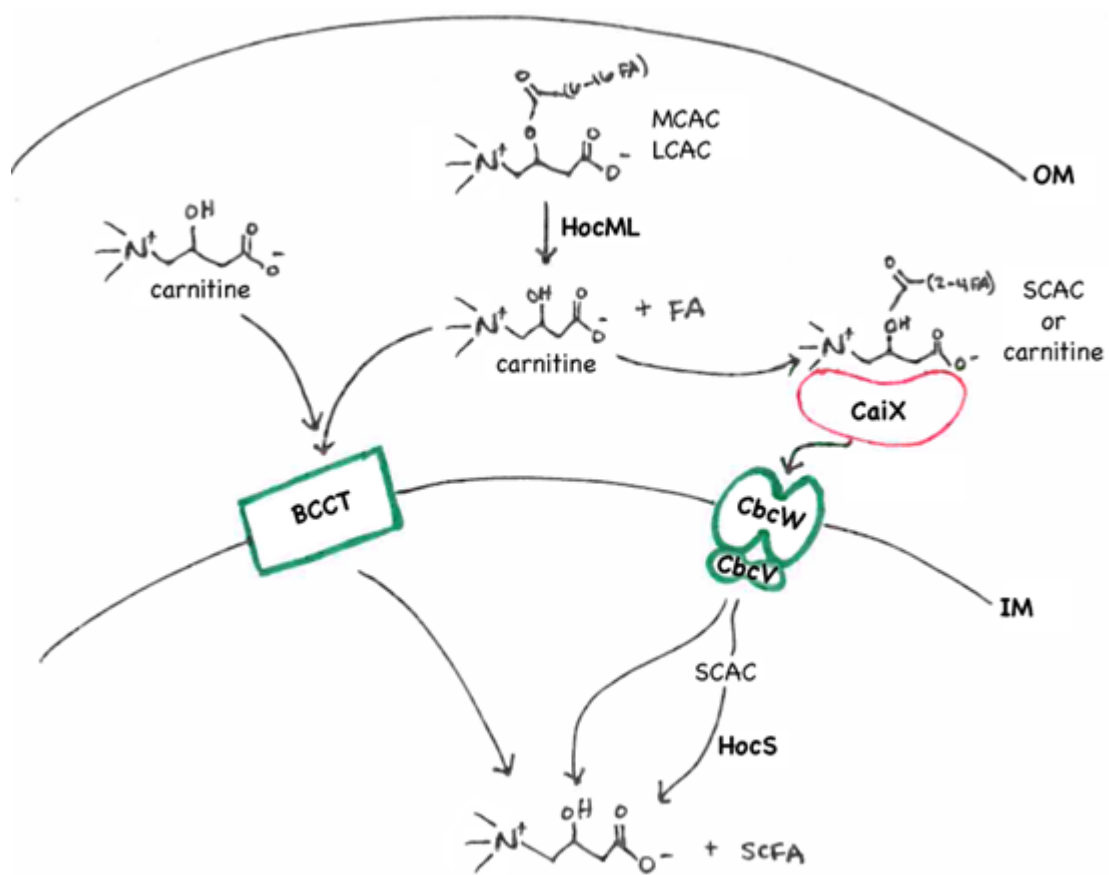


Figure 7. Model of *P. aeruginosa* transport of acylcarnitines.



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## **CHAPTER 4:**

### **REGULATION OF CARNITINE METABOLISM IN *PSEUDOMONAS***

### ***AERUGINOSA***

#### 4.1. Abstract

*Pseudomonas aeruginosa* is found in numerous environments and is an opportunistic pathogen affecting those who are immunocompromised. The large metabolically diverse genome and regulatory network that *P. aeruginosa* possesses make it versatile to adapting to various environments. We are interested in how *P. aeruginosa* senses and responds to host-derived compounds. Previously we identified CdhR and GbdR, members of the AraC transcription factor family that regulate catabolism of the quaternary amine compounds carnitine and glycine betaine (GB), respectively. Our goal was to further characterize regulation of carnitine catabolism by the TF CdhR. Here we report that CdhR binds in a concentration dependent manner to the enhancer site near the carnitine operon promoter (*P<sub>caiXcdhCABhocS</sub>*). We identified the CdhR binding site and compared with the known GbdR binding consensus, to show that the two binding sites overlap in the *caiX-cdhR* intergenic region. Carnitine catabolism is repressed by glucose and GB at the transcriptional level. Furthermore, using two different *cdhR* translational fusions we show that CdhR enhances its own expression and that GbdR contributes to *cdhR* expression by enhancing the level of basal expression.

#### 4.2. Introduction

*Pseudomonas aeruginosa* is a Gram-negative bacterium found in soil, water, on skin and on surfaces, such as bed rails, catheters, and other medical devices [1, 2]. From the sites it can transition into being an opportunistic human pathogen that is the leading cause of infection in people with severe burns [3], and those with lung diseases like

chronic obstructive pulmonary disease, ventilator-associated pneumonia, and cystic fibrosis, resulting in increased morbidity and mortality [4-7]. With antibiotic resistance on the rise [8], *P. aeruginosa* is difficult to treat and is a burden on our health care system [9]. The ability to transition from one environment to the next and cause disease is, in part, due to its large genome, with 5% of genes predicted to encode proteins related in carbon metabolism [10].

Carnitine and *O*-acylcarnitines are quaternary amine compounds that are abundant in host tissues and shuttle fatty acids in and out of the mitochondria for  $\beta$ -oxidation in animals [11]. There are no animal enzymes that can breakdown carnitine, consequently any degradation is due to bacteria [12], like *P. aeruginosa* [13]. No *de novo* synthesis of carnitine has been demonstrated in bacteria [14], therefore *P. aeruginosa* acquires it from the environment by import through the ABC transporter CbcWV [15]. *P. aeruginosa* contains a carnitine catabolism operon *caiXcdhCABhocS*, which encode enzymes to degrade short-chain acylcarnitines and carnitine for osmoprotection, and its catabolic product glycine betaine (GB) induces the virulence factor phospholipase C, *plcH* and be used as sole carbon, nitrogen, and energy source [16-19]. Medium- and long-chain acylcarnitines, with the exception of octanoylcarnitine, can be used as sole carbon sources as well, [16].

The ability to adapt to numerous environments can be attributed to the large regulatory repertoire that *P. aeruginosa* possesses. More than 9% of the genome is dedicated to transcriptional regulation, including regulation of metabolism, giving the bacterium metabolic flexibility [10]. One such regulator is CdhR, which is divergently

transcribed from the carnitine catabolism operon and is required for growth on carnitine and induction of the carnitine operon [17]. Carnitine degradation leads to the formation of glycine betaine, whose catabolism is regulated by GbdR [20]. GbdR and CdhR not only regulate catabolism of quaternary amine compounds but both are AraC-family transcription factors that belong to the same GATase-1 subfamily and are highly similar in sequence (62% positive and 44% identities) [21].

In this study we expand our understanding of the regulation of carnitine catabolism, by identifying the enhancer site, essential residues, and showing catabolite repression from glucose and glycine betaine function at the level of transcription of the carnitine operon. Although an additional CdhR binding site was found upstream of *cbcWV* to which CdhR binds in a concentration dependent manner, we established that transcription from this locus is controlled solely by GbdR in response to glycine betaine. GbdR is also capable of binding the intergenic region of *caiX-cdhR* in an orientation suggesting regulation of *cdhR*. Additionally, CdhR positively regulates its own expression in the presence of carnitine but represses basal expression in the absence of ligand, however GbdR alleviates this repression. We propose that there is a hierarchy to CdhR and GbdR binding at their shared binding site with and without ligand.

### **4.3. Materials and methods**

#### **4.3.1. Strains and growth conditions**

*Pseudomonas aeruginosa* strains PAO1 and PA14 were maintained on PIA plates or Lennox broth (LB) liquid, and when necessary 50  $\mu\text{g ml}^{-1}$  or 40  $\mu\text{g ml}^{-1}$

gentamicin was added to the media, respectively. *Escherichia coli* NEB5 $\alpha$  or T7 Express *E. coli* (NEB C3016) were maintained on LB plates with 10  $\mu\text{g ml}^{-1}$  gentamicin, LB liquid with 7  $\mu\text{g ml}^{-1}$  gentamicin, LB plates/liquid with 125  $\mu\text{g ml}^{-1}$  carbenicillin, or LB plates/liquid with 100  $\mu\text{g ml}^{-1}$  kanamycin.

#### **4.3.2. Deletion and complementation constructs**

A clean deletion of *PA5389* (*cdhR*) in PAO1 was made in the wild type strain MJ79. The upstream and downstream regions of *PA5389* were PCR amplified from PAO1 genomic DNA with the primers 5389GOIF1KpnI, 5389SOEGOIR1, 5389SOEGOIF1, and 5389GOIR1BamHI. Splice overlap extension PCR product was ligated into the Zero Blunt™ plasmid pCR (Invitrogen), excised with EcoRI, and ligated into similarly cut pMQ30 to generate pJAM90. Donor *E. coli* S17 $\lambda$ pir carrying pJAM90 were mated with PAO1 and screened as previously described [16], generating strain JM236. Deletion strain was verified by lack of growth on carnitine and by PCR.

To make a double mutant of *cdhR* and *gbdR* in PA14, the  $\Delta$ *gbdR* PA14 strain MJ26 was used as the recipient strain for mating with *E. coli* S17 $\lambda$ pir carrying pJAM90 as described above. Double mutant strain was verified by PCR.

#### **4.3.3. Construction of transcriptional constructs**

To determine the promoter of *PA5388* (*caiX*) four different truncations of the upstream region of *caiX* were cloned as transcriptional reporter constructs. pMW79 was used as the PA14 PCR template for amplifying the upstream region of *caiX*.



PA5388promR is the reverse primer used in all four reactions and the forward primers are PA5388promF1, PA5388promF2, PA5388promF3, and PA5388promF4. The four PCR products were ligated into the Zero Blunt vector pCR, digested with KpnI and HindIII, and ligated into a similarly cut pMW5 to yield four different P5388*lacZYA* transcriptional fusions (pJAM22-pJAM25). Each was electroporated into PA14WT and selected on PIA gentamicin plates.

To assess which residues were essential for induction of *caiX*, seven different transcriptional reporters were constructed by amplifying the *caiX* binding site from pMW79 using the 5388promR primer and seven different forward primers: P5388pos, P5388mut1, P5388mut2, P5388mut3, P5388mut4, P5388mut5, P5388mut6. PCR products were digested with HindIII and KpnI, and ligated into similarly cut pMW5. The resulting plasmids pJAM122-pJAM127 and pJAM130 were electroporated into PA14WT and selected on PIA gentamicin.

#### **4.3.4. Construction of translational constructs**

To determine translational control of *cdhR* a divergent fluorescent translational reporter with YFP and CFP on either side of the intergenic region of *PA5388* and *PA5389* was made. YFP was amplified using primers YFP F Kpn Sal and YFP R HindIII from the template pUC18miniTn7T-Gm-*eyfp* [22]. The YFP fragment was ligated into pCR Zero Blunt plasmid, digested with HindIII and KpnI, and subsequently ligated into similarly cut pMQ80. The resulting plasmid was digested with EcoRI and SacI and ligated with a *P. aeruginosa* codon-biased CFP made by IDT, which was cut with EcoRI and SacI, to

generate pJAM76. The *PA5388-PA5389* intergenic region was amplified using primers 88-89YC-DR#2ycF and 88-89YCDR#2ycR. pJAM76 was then digested with SalI to linearize the plasmid for recombination of the *PA5388-PA5389* intergenic regions using yeast cloning in *Saccharomyces cerevisiae* via the method of Shanks et al [23]. The resultant constructed plasmid was digested with HindIII and ligated into similarly cut pUC18-mini-Tn7T-Gm yielding pJAM86, which was coelectroporated with pTNS2 to insert CFP-PA5388-PA5389-YFP onto the chromosome at the *attTn7* site of strains PAO1 wild type, PAO1 $\Delta$ *cdhR* and PAO1 $\Delta$ *gbdR*.

A *PA5389* translational *lacZ* fusion was made by inserting a gBlock™ made by IDT that has 320 base pairs of the upstream region of *PA5389* to the translational start site of *PA5389* and then from the translational start site of *lacZ* to + 850 bases in the *lacZ* gene into pJAM131. pJAM131 was built by PCR amplifying the C-terminal end of *lacZ* with primers 2-lacZCtermFhindcla and 2-lacZCtermRsmakpn, digested with HindIII and KpnI, and ligated into a similarly cut pUCP22. The gBlock was digested with HindIII and ClaI and ligated into similarly cut pJAM131 to make pJAM135.

#### **4.3.5. Cloning and expression of MBP-CdhR**

The expression plasmid pJAM50, a maltose binding protein (MBP) fusion to the amino-terminus of CdhR was made by PCR amplifying *cdhR* with primers 5389Mal-C2XF and 5389Mal-C2XR, and the product was ligated into Zero Blunt pCR vector. *cdhR* was extracted with EcoRI and HindIII and ligated into the similarly cut pMal-C2X

(NEB), transformed by electroporation into T7 Express *E. coli* (NEB C2566), and selected on LB carbenicillin to make strain JM153.

To express MBP-CdhR, 1L of JM153 was grown at 37 °C in LB with 75 µg ml<sup>-1</sup> to an OD<sub>600</sub> of 0.4. The culture was induced with 1 mM IPTG and grown for an additional 3 hours. Cells were collected by centrifugation and resuspended in 20 mM Tris HCl, 150 mM NaCl, pH 7.4 with 1X halt protease inhibitor cocktail (Thermo) and lysed by French press. DNaseI was added to the lysate and incubate room temperature for 15 minutes. Samples were centrifuged at 13K rpm for 30 minutes at 4 °C to separate the soluble and insoluble fractions. The soluble fraction was filtered through a 0.22 µm filter and applied to an Affi-Gel Heparin Gel (BioRad) column. The column was washed with column buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4 in 1XPBS) and MBP-CdhR was eluted from the column with column buffer plus 1.5 M NaCl. Fractions containing MBP-CdhR were run on a 12% SDS denaturing gel and stained with coomassie brilliant blue (Thermo) to verify purity and determine which fractions to use. Elutions containing MBP-CdhR were dialyzed in a 20K MWCO slidealyzer (Thermo) overnight at 4 °C in buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4).

#### **4.3.6. Primer extension**

Transcriptional start site of *caiX* and *cdhR* were determined by growing PA14WT carrying pMW79 [17] or PA14WT carrying pJAM135, respectively, in MOPS, 20 mM pyruvate, 5 mM glucose, and 20 µg ml<sup>-1</sup> gentamicin overnight at 37 °C. Cells were washed, resuspended to an OD<sub>600</sub> nm of 0.3 in MOPS supplemented with 20 mM L-

carnitine or pyruvate and  $20\ \mu\text{g ml}^{-1}$  gentamicin in a 12 well-plate, and grown for 7 hours at  $37\ ^\circ\text{C}$  while shaking. Cells were collected by centrifugation and RNA was purified per the instructions of the RNeasy Mini Kit (Qiagen). Primer extension was completed using the purified RNA as the template for cDNA generation using the Superscript II RT (Invitrogen) and the 5'-fluorescein-labeled primers 5388primerextension or 5389primerextension. The cDNA products were analyzed by capillary electrophoresis on an ABI to determine the size of the DNA fragment of the induced carnitine sample versus the uninducing pyruvate and by comparing it to a known DNA size standard.

#### **4.3.7. Beta-galactosidase assays**

Strains were grown overnight at  $37\ ^\circ\text{C}$  in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and  $20\ \mu\text{g ml}^{-1}$  gentamicin. Cells were collected by centrifugation, washed, resuspended in MOPS and inoculated into MOPS with 20 mM pyruvate,  $20\ \mu\text{g ml}^{-1}$  gentamicin, and with or without 1 mM inducing compound carnitine. Induction was carried out for 4 hours at  $37\ ^\circ\text{C}$ , and  $\beta$ -galactosidase assays were performed according to Miller [24].

#### **4.3.8. Catabolite repression**

PA14WT carrying pJAM22 was grown overnight at  $37\ ^\circ\text{C}$  in MOPS with 20 mM pyruvate, 5 mM glucose, and  $20\ \mu\text{g ml}^{-1}$  gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS, and inoculated into MOPS with 20 mM pyruvate and  $20\ \mu\text{g ml}^{-1}$  gentamicin at a final  $\text{OD}_{600}$  of 0.05. All samples had 1 mM

of the inducing compound carnitine except for the noninducing control (pyruvate alone). Catabolite repression samples had either glucose at 2 mM, 4 mM, or 10 mM or glycine betaine at 20 mM or 40 mM. Cultures were induced for 4 hours at 37 °C, and  $\beta$ -galactosidase assays were performed according to Miller [24].

#### **4.3.9. Electrophoretic Mobility Shift Assays (EMSA)**

To determine binding of MBP-CdhR or MBP-GbdR to promoters, EMSAs were performed as previously described [21]. Briefly, *caiX*, *dhc*, *cbcX*, *cdhR*, or *cdhR* mutant promoter DNA fragments were made by PCR amplification from PA14 genomic DNA template with primers listed in the EMSA primer section in Table 2. The DNA probes were dialyzed in 1/4 TE on a 0.025  $\mu$ m filter for 20 minutes and verified by PCR. EMSAs were conducted by following the LightShift Chemiluminescent EMSA kit instructions (Pierce) as modified by Hampel et al [21]. Samples were electrophoresed on 5% TBE nondenaturing gels, transferred to BioDyne-B nylon membrane (Thermo), and detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo) per manufacturer's instructions.

#### **4.3.10. DNaseI footprinting assay**

DNaseI footprinting was performed as described previously [21]. Briefly, the target DNA was made by labeling the 5' end of one the primers PA5388promR or PA5388promF2 with  $^{32}$ P using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP, and amplifying the PA14 *caiX* upstream region. The radiolabeled PCR product was purified from a 5%

polyacrylamide Tris-borate-EDTA gel. The MBP-CdhR footprinting assay was conducted as in Brenowitz [25] and Hampel et al [21].

#### **4.3.11. Fluorescent microscopy**

*P. aeruginosa* PAO1 strains WT,  $\Delta cdhR$ , and  $\Delta gbdR$  with the divergent fluorescent reporter *PA5388-PA5389* intergenic region CFPYFP-DR2 (pJAM86) at the *attTn7* site, were grown overnight in MOPS with 20 mM pyruvate and 5 mM glucose at 37 °C. Cells were centrifuged, washed, and resuspended in MOPS to an OD<sub>600</sub> of 1.0. A 1/20 dilution was made and 1 µl was placed on the center of the agar pad (1.5% low melting agarose, MOPS, 20 mM pyruvate, and with or without 1 mM carnitine). The agar pad was placed upside down on a 50 mm glass bottom cell culture dish (Warner Instruments). Samples were imaged on a Nikon Ti-E, every 10 minutes for 6 hours, at 32 °C and exposure time was 100 msec for phase and 500 msec for YFP. Image stacks were imported into Fiji 2.0.0-rc-29/1.49s [26] using the Bio-Formats Importer 5.1.1 [27]. A rolling variance filter on the contrast channel was used to define background areas, and after an additional threshold was applied, the find maxima macro was used to define cell areas. Using masks from the contrast channel mean pixel intensity measurements were taken from the YFP channel. A framewise background intensity correction was performed for each cell using R 3.2.0 [28].

## 4.4. Results

### 4.4.1. Promoter mapping reveals the CdhR binding site.

The carnitine catabolism operon, *caiX-cdhCAB-hocS* (Fig. 1), encodes proteins that are responsible for the hydrolysis of short-chain acylcarnitines [16] and degradation of carnitine to glycine betaine [17, 29]. *cdhR* is divergently transcribed from the carnitine catabolism operon (Fig. 1), and was previously shown to be required for induction of *caiX* and growth on carnitine [17]. To expand upon this basic understanding of CdhR's role in carnitine catabolism, we determined the binding site of CdhR by promoter mapping of *PcaiX* using four sequentially shorter *lacZYA* transcriptional reporters. The constructs end at base pair (bp) -22 from the *caiX* translational start site and begin at bp -424 (pJAM22), bp -327 (pJAM23), bp -241 (pJAM24), and bp -137 (pJAM25)(Fig. 2). In the presence of carnitine all constructs except pJAM25 were induced, indicating that the binding site of CdhR is between bp -241 and -137 (Fig. 2). The binding site may also overlap with bp -137 with a partial site not sufficient to induce of *caiX*.

Primer extension was used to define the transcriptional start site of *caiX* and *cdhR*. Plasmids containing the target regions were used to increase RNA copy number, particularly for *cdhR*, as native transcripts are at low abundance [30]. The length of the *caiX* primer extension product place the transcriptional start site 25 bases upstream of the translational start site at a thymine residue. The length of the *cdhR* primer extension primer placed the transcriptional start site 63 bases upstream of the translational start site at a cytosine.

#### **4.4.2. Glucose and glycine betaine repress the transcription of *caiX*.**

In *P. aeruginosa* the enzymatic activity of carnitine dehydrogenase (CDH) declines until no longer detectable if the cells are switched from carnitine to the preferred carbon source glucose, and the activity oscillates when carnitine is the sole carbon source. It is thought that oscillation occurs when the catabolic product glycine betaine is produced, resulting in initiation of a negative feedback oscillation loop [31]. To assess catabolite repression, we used the *caiX-lacZYA* transcriptional reporter (pJAM22) to determine if repression is transcriptionally regulated. Maximal catabolite repression is seen at 4 mM glucose, while GB represses transcription to a lesser extent and at a higher concentration (Fig. 3).

#### **4.4.3. MBP-CdhR binds the *caiX* upstream activating site in a concentration-dependent manner.**

Based upon CdhR-dependent transcriptional induction of *caiX*, we assayed the capability of purified MBP-CdhR to bind to the activating site (UAS) of *caiX*. Using biotin labeled *caiX* UAS as the DNA probe and purified MBP-CdhR, electrophoretic mobility shift assays revealed that MBP-CdhR binds the *caiX* UAS in a concentration dependent manner (Fig. 4 lanes 1-4). The binding interaction between MBP-CdhR and *caiX* probe can be competed with unlabeled *caiX* probe (lane 5) and MBP-CdhR does not shift a nonspecific UAS, *dhcA* (lanes 6-8). These data demonstrate that MBP-CdhR binds specifically to the *caiX* UAS (Fig. 4).



#### **4.4.4. Identification of the CdhR binding site sequence and bases required for induction of *caiX*.**

Promoter mapping and EMSAs determined the region of DNA essential binding of CdhR and transcriptional induction of *caiX*. To further characterize the CdhR DNA contact site and binding site sequence within the *caiX* UAS, we employed DNaseI footprinting. Purified MBP-CdhR and radiolabeled *caiX* UAS demonstrated the characteristic and site specific protection of DNA as protein concentration increased. Comparing the zone of protection to the A+G ladder we were able to identify the CdhR contact region, stretching 34 bases (Fig. 5). The CdhR binding site reveals the half sites each contain the sequence GGTCGC with 15 bases in between, which is very similar to the binding sites of two other *P. aeruginosa* AraC transcription factors, GbdR [21] and ArgR [32]. In order to further characterize the CdhR binding site, we made mutations in the distal repeat by systematically changing partially overlapping two bases to adenosines (pJAM122-127). Using LacZ as the reporter, induction of the mutated *caiX* binding sites was assessed to determine that the second guanine residue in the repeat is essential for *caiX* expression but complete abolishment of expression requires an additional transversion mutation of the adjacent locus thymine residue (Fig. 5).

#### **4.4.5. CdhR binds, but does not regulate the ABC transporter *cbcXWV*.**

After establishing the CdhR binding sequence, GGTCGC-[N15]-GGTCGC, we wanted to identify if the sequence was located elsewhere in the *P. aeruginosa* PAO1 genome. Using the DNA motif search tool from the Pseudomonas Genome Database

website [33], only two sites were identified within intergenic regions, *caiX-cdhR* and *cbcX-sdaB*, both of which are involved in carnitine metabolism [15, 17]. CbcXWV is an ABC transporter and the core transporter proteins CbcWV are required for growth on carnitine [15]. A MBP-CdhR EMSA with the *cbcX* promoter DNA probe shows MBP-CdhR binding in a concentration dependent manner (Fig. 6A), but the qRTPCR data reveals carnitine is not sufficient for induction of *cbcX* (Fig. 6B). We used the *cdhCA* deletion to eliminate production of GB from carnitine (Fig. 1) [17]. In order to induce *cbcX*, GB must be produced to allow induction of *cbcX* transcription via GbdR [21].

#### **4.4.6. GbdR binds the intergenic region of *caiX-cdhR*.**

The ability of CdhR to bind a known member of the GbdR regulon (*cbcX*) [21, 34], the detection of the GbdR binding consensus in the *caiX-cdhR* intergenic region [21], and the overlapping position of the CdhR and GbdR binding sites led us to further investigate the role of GbdR in carnitine regulation. We predicted that GbdR would be able to bind the *cdhR* UAS and an EMSA with purified MBP-GbdR in association with the *cdhR* UAS showed that MBP-GbdR binds the *cdhR* UAS in a concentration dependent manner (Fig. 7A). When the GbdR conserved residues CG in the distal half site were mutated to AA, binding was lost (Fig. 6), as seen with the *plcH* and *choE* mutated distal half site [21]. Since GbdR binds the *caiX-cdhR* intergenic region, induction of *caiX* (pJAM22) was assessed in relation to GbdR. A *gbdR* mutant has significantly less induction of *caiX* but this defect is likely due to a defect in carnitine import (as discussed in chapter 3). In addition, these data suggest that GbdR is not the

transcriptional activator of *cdhR* since *caiX* is induced in a *gbdR* mutant, whereas in a *cdhR* mutant, *caiX* induction is completely abolished.

#### **4.4.7. CdhR is required for induction of *cdhR* expression.**

After establishing that CdhR and GbdR bind the intergenic region of *caiX-cdhR*, we wanted to determine expression of *cdhR* using a translational reporter in relation to CdhR and GbdR. Using two different translational fusions, one carried on a plasmid and one integrated into the chromosome at the *attTn7* site, it became apparent that CdhR has a role in its own expression. In wild type carnitine increased expression of *cdhR* compared to the basal expression level (uninduced) (Fig. 8). In the absence of *gbdR*, carnitine still induces *cdhR*, but basal expression of *cdhR* is decreased compared to wild type and a *cdhR* deletion, suggesting that GbdR functions to relieve repression at this locus (Fig. 8). These data analyzing the expression of *cdhR* may represent a hierarchy of the transcription factors GbdR and CdhR which we will discuss below.

### **4.5. Discussion**

The metabolic diversity of *P. aeruginosa* is a product of the immense regulatory network encoded in the genome and it is this network of transcription factors (TF) that are a critical mechanism for sensing environmental cues [35]. One such family of transcription factors that controls carbon metabolism and virulence is the AraC transcription factor family. In this study we expand on our understanding of carnitine catabolism and how two AraC transcription factors, CdhR and GbdR, whose metabolic

pathways converge at glycine betaine (Fig. 1), are intertwined. We identified the CdhR enhancer site and through reporter and DNA binding assays reveal this site as an additional GbdR binding site. Through reporter fusions we were able to show that CdhR regulates its own expression is responsive to carnitine. We also show that GbdR binding to *caiX-cdhR* region assists in regulating carnitine catabolism by inhibiting repression of *caiX* and *cdhR* transcription and maintaining a basal *cdhR* expression level.

The first question we addressed was how CdhR binds and regulates the carnitine operon. CaiF in *E. coli* is the only other carnitine regulator that has been characterized to date, and is a degenerate AraC TF that lacks the traditional N-terminal domain but maintains the helix-turn-helix DNA binding domains [36]. However, CaiF is transcribed in the opposite direction from the carnitine metabolism operon (*caiTABCDE*) and binds to inverted repeats in the enhancer site [36, 37]. *P. aeruginosa* and other Gram-negative bacteria that contain a carnitine catabolism operon capable of generating glycine betaine are organized in similar fashion to one another with an AraC-family transcription factor divergently transcribed from the catabolic operon [38]. CdhR binds to direct repeats and the binding site is upstream of the *caiX* promoter (Fig. 5), categorizing CdhR as a class I activator that requires both half sites for induction of *caiX* (Fig. 2).

Kleber and Aurich analyzed the activity of carnitine dehydrogenase (CDH) with respect to glucose and glycine betaine and showed that glucose, as a preferred carbon source, is catabolite repressive [31], whereas glycine betaine leads to repression of CDH activity resulting in oscillations of activity as carnitine is catabolized to GB [31]. Our data shows that repression by glucose and glycine betaine is controlled at the level of

transcription (Fig. 3). Negative feedback by glycine betaine is likely GbdR dependent based on GbdR's capability to bind the *caiX-cdhR* intergenic region and GbdR's responsiveness to GB [39]. *P. aeruginosa* maintains intracellular glycine betaine pools and GbdR fine tuning carnitine catabolism may be directly related to sustaining the homeostatic levels of glycine betaine, as the pool has a physiological impact on nutrients, osmoprotection and virulence [40]. Chapter 5 address experiments to test whether this is the case.

We performed an alignment using the *Pseudomonas.com* DNA motif search tool of the newly identified CdhR binding sequence to the PAO1 genome and identified two intergenic regions: *caiX-cdhR* and *cbcX-sdaB* [33]. This led us to investigate if CdhR contributes to the regulation of carnitine import by the ABC transporter *cbcXWV*, which is required for growth on carnitine [15]. Even though CdhR binds to the *cbcX* upstream region *in vitro*, it does not contribute to *cbcX* expression (Fig. 6). We propose that an unknown BCCT transporter imports carnitine, it is degraded to GB, which drives expression of *cbcXWV* in a GbdR dependent manner allowing for a larger flux of carnitine which is needed for growth [15]. This is similar to the mechanism of choline import for *cbcXWV* induction, termed priming [34].

We previously characterized the GbdR regulon and identified the intergenic region of *caiX-cdhR* to have a GbdR binding site [21]. Here we report that GbdR binds the *caiX-cdhR* intergenic region *in vitro* and the conserved CG residues are necessary for GbdR binding, as mutating the residues to AA results in loss of binding (Fig. 7). Based upon the conserved residues being located in the distal half site [21], the orientation of

GbdR binding is likely oriented towards *cdhR* activation and not *caiX*. Interestingly, the GbdR and CdhR binding sites overlap in the *caiX-cdhR* intergenic region, which led us to hypothesize that GbdR has a role in regulating carnitine catabolism and in particular *cdhR*.

Based on what we know about GbdR and what we presented here, we propose a schematic for the interplay of the two AraC TFs and how they regulate carnitine catabolism. In the absence of carnitine, CdhR binds to its target site in a manner that inhibits *cdhR* expression. GbdR can compete for the binding sites and prevent CdhR-dependent inhibition by CdhR, based on comparisons of basal expression levels (Fig. 8). Analyzing the DNA sequence up and downstream of the CdhR binding site reveals multiple CdhR half sites. These half sites could be participating in CdhR inhibition of basal expression. CdhR inhibition of its own expression may be similar to the AraC ‘light-switch’ mechanism (Figure in Chapter 1). In the absence of ligand, one AraC DNA binding domain binds an operator half site promoting DNA looping and prevents expression of the arabinose operon *araBAD*. Upon ligand binding AraC undergoes a conformational change promoting binding of both half sites of the enhancer and inducing transcription [41]. Another possibility is that CdhR oligomerizes along the DNA nucleated at these half sites and dampens *cdhR* basal expression. In this model, GbdR could compete for binding, supported by the lower basal expression in a *gbdR* mutant, where CdhR is binding and inhibiting *cdhR* expression. Furthermore, CdhR binding of carnitine causes a conformational change, allowing CdhR to increase expression of *cdhR* and *caiX* (Fig. 8). As the catabolic product GB builds up, the cell controls the flux of

carnitine catabolism by GB-dependent transcriptional repression, which is likely regulated by GbdR. Fine tuning of this pathway is predominately controlled by GbdR, a principal regulator.

In conclusion, we show regulation of carnitine catabolism is controlled by two transcription factors that contribute to fine tuning of the system depending on when and if ligands are present. We put forward a hierarchy of regulation, where one transcription factor trumps the next depending on ligand:  $\text{GbdR+GB} > \text{CdhR+carnitine} > \text{GbdR} > \text{CdhR}$ . This system has likely evolved to control nutrient acquisition, virulence, GB pools, and possibly other metabolic bi-products like formaldehyde and peroxide, which are involved in carnitine and GB metabolism.

## 4.6. Tables

**Table 1. Strains and plasmids used in this study**

Strain or plasmid	Genotype or description	Reference
<u><i>P. aeruginosa</i> PAO1</u>		
MJ79	wild type	[10]
MJ80	$\Delta gbdR$	[20]
JM236	$\Delta cdhR$	This study
JM253	wild type::88-89intYFPCFP-2	This study
JM339	$\Delta cdhR::88-89intYFPCFP-2$	This study
JM340	$\Delta gbdR::88-89intYFPCFP-2$	This study
<u><i>P. aeruginosa</i> PA14</u>		
MJ101	wild type	[42]
MJ11	$\Delta cdhR$	[17]
MJ26	$\Delta gbdR$	[20]
MJ262	$\Delta cdhCA$	[17]
JM179	$\Delta cdhR \Delta gbdR$	This study
<u><i>E. coli</i></u>		
MJ340	wild type S17 $\lambda$ pir	
DH5 $\alpha$	NEB C2987	NEB
T7Express	NEB C3016	NEB
<u>Plasmids</u>		
pMQ30	suicide vector, Gm <sup>r</sup>	[23]
pMQ80	high-copy number <i>Pseudomonas</i> vector, Gm <sup>r</sup>	[23]
pMal-C2X	T7 Expressing vector, MBP N-terminal tag, Amp <sup>r</sup>	NEB
pTNS2	plasmid carrying the <i>attTn7</i> transposase	[43]
pUC18-mini-Tn7T-Gm	Gm <sup>r</sup> on mini-Tn7T	[22]
pUC18-mini-Tn7T-Gm- <i>eyfp</i>	Gm <sup>r</sup> on mini-Tn7T with YFP	[22]
pUCP22	high copy # <i>Pseudomonas</i> stabilization vector, Gm <sup>r</sup>	[44]
pMW5	<i>lacZYA</i> in pUCP22	[39]
pMW79	PA14 genomic clone of <i>PA5380-PA5389</i> in pMQ80	[17]
pJAM22	promoter <i>caiX-lacZYA</i> transcriptional fusion-A	This study
pJAM23	promoter <i>caiX-lacZYA</i> transcriptional fusion-B	This study
pJAM24	promoter <i>caiX-lacZYA</i> transcriptional fusion-C	This study
pJAM25	promoter <i>caiX-lacZYA</i> transcriptional fusion-D	This study
pJAM50	<i>PA5389</i> in pMal-C2X	This study
pJAM76	YFP-CFP in pMQ80	This study
pJAM86	CFP <i>PA5388-PA5389</i> intergenic region YFP in pUC18mini, DR2	This study



pJAM90	<i>PA5389</i> deletion construct in pMQ30	This study
pJAM122	promoter <i>caiX-lacZYA</i> transcriptional fusion	This study
pJAM123	promoter <i>caiX-lacZYA</i> mut 1 transcriptional fusion	This study
pJAM124	promoter <i>caiX-lacZYA</i> mut 2 transcriptional fusion	This study
pJAM125	promoter <i>caiX-lacZYA</i> mut 3 transcriptional fusion	This study
pJAM126	promoter <i>caiX-lacZYA</i> mut 4 transcriptional fusion	This study
pJAM127	promoter <i>caiX-lacZYA</i> mut 5 transcriptional fusion	This study
pJAM130	promoter <i>caiX-lacZYA</i> mut 6 transcriptional fusion	This study
pJAM131	C terminus of <i>lacZ</i> in pUCP22	This study
pJAM135	<i>PcdhRlacZYA</i> in pUCP22	This study

**Table 2. Primers used in this study**

**Primers 5' to 3'**

Deletion constructs

5389GOIF1KpnI	ATAGGGTACCgaagaacaccacccactgct
5389SOEGOIR1	aagtacgaaggcgactcgaCCATGGagaagcccattaccgagaagc
5389SOEGOIF1	gcttctcggtaatgggcttctCCATGGtcgagtcgccttcgtactt
5389GOIR1BamHI	ATCGTCTTCGCTGTTTTTCC

Protein expression construct

5389Mal-c2xF	GCATCAgaattcTCCCAGGACTTCTGGTTTCT
5389Mal-c2xR	GCATCAaagcttTCAGCCTCGCTCAGCTCGA

Primer extension

5388primerextension	5'-fluorescein 6 fam-ACTGGCCAGGATCAGCAGG
5389primerextension	5'-fluorescein 6 fam-AGACAGTATCGGCCTCAGGAA

EMSA probes

PA5388promF3	aagcttGTGCCAGCGGTAGAGGTC
PA5388promR	TGAggtaccTTGATTGTTTTTCTGCGAGGT
PA5388promRbiot	biotin-TTGATTGTTTTTCTGCGAGGT
5389EMSA-F	ATGAaagcttGCAGCAGGAGAAACCAGAAG
5389EMSA-R-biot	biotin-TTGATTGTTTTTCTGCGAGGT
5389EMSA-Mut3F	GGACGGCGGCGAAGCGCACTGCGAAGACC
cbcXprom-F	CCGGCAAAGACCACTATGAT
cbcXprom-R-biot	biotin-GAACTCCTCTGCAGGGTAAGG
dhcXprom-F-biot	biotin- GAGGCTTTCCTCCAGGCTCT
dhcXprom-R	GGATggtaccCTCTTCCGGCTCTTGTGATT
dhcXprom-F	GAACTCCTCTGCAGGGTAAGG

Transcriptional reporters

PA5388promR	TGAggtaccTTGATTGTTTTTCTGCGAGGT
PA5388promF1	ATGAaagcttACAGCAGGTGCGCTTTTCTT
PA5388promF3	aagcttGTGCCAGCGGTAGAGGTC
PA5388promF2	ATGAaagcttGCAGCAGGAGAAACCAGAAG
PA5388promF4	aagcttCTGCAGTGCAAGAGCTGGT
P5388pos	ATGAaagcttCGCTTGGCAATGGCCAGGTCGCT
P5388mut1	ATGAaagcttCGCTTGGCAATGGCCAAATCGCT
P5388mut2	ATGAaagcttCGCTTGGCAATGGCCAGAACGCT
P5388mut3	ATGAaagcttCGCTTGGCAATGGCCAGGAAGCT
P5388mut4	ATGAaagcttCGCTTGGCAATGGCCAGGTAAGT
P5388mut5	ATGAaagcttCGCTTGGCAATGGCCAGGTCAAT

P5388mut6

ATGAaagcttCGCTTGGCAATGGCCAGATCGCT

Translational reporters

2-lacZCtermFhindcla

2-lacZCtermRsmakpn

YFP R HindIII

YFP F Kpn Sal

88-89YC-DR#2ycF

88-89YC-DR#2ycR

GCaagcttATTATCGATGAGCGTGGTGGTTATGC

cggtagccggggatccTTATTTTGGACACCAGACC

GCATCAaagcttATTACTTGTACAGCTCGTCCA

GACAGcggtagcAATCgtcgacCATatgctgagcaagggcgagg

gggcaccaccccggtgaacagtcctcgcccttgctcagcat-

GGGGCGCTCCGGGGTTGA

cggcaccacgcgggtgaacagtcctcgcccttgctcagcat-

CGGTCTCCCCTCGTGCGG

## 4.7. Figures

**Figure 1. Diagram of the *P. aeruginosa* PAO1 carnitine catabolism operon and the catabolic pathway.** (A) Arrows represent the individual open reading frames of the carnitine catabolism operon and the regulator *cdhR*. Below the arrow is the designated gene name. (B) A diagram of the converging carnitine and choline catabolism pathways. Black arrows represent an enzymatic step in the catabolic pathway and the gene names are italicized below. The blue arrows represent positive regulation by either CdhR or GbdR and the T bar represents repression by BetI.

**Figure 2. Promoter mapping of the upstream region of *caiX* exposes the CdhR binding site.** Transcriptional fusions of *lacZ* to the upstream region of *caiX* starts at base pair -22 from the *caiX* translational start site and end at base pair -424 for pJAM22, -327 for pJAM23, -241 for pJAM24, and -137 for pJAM25. *P. aeruginosa* PA14 carrying pJAM22, pJAM23, pJAM24, or pJAM25 were grown in MOPS with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and induced with 1 mM carnitine. The cultures were subjected to Miller assays to assess relative induction of the different *caiX* transcriptional fusions. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments.

**Figure 3. *caiX* transcription is repressed by glucose and glycine betaine. *P. aeruginosa* PA14 with *caiX-lacZ* (pJAM22) was grown in MOPS, 20 mM pyruvate and 20  $\mu\text{g ml}^{-1}$  gentamicin. The positive control and samples have 1 mM carnitine. All**

Catabolite repression samples have additional compound added; 10 mM, 20 mM, 30 mM or 40 mM glycine betaine (pH 7), or 2 mM, 4 mM or 10 mM glucose. Cultures were induced for 4 hours prior to running a Miller assay. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments. \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

**Figure 4. MBP-CdhR binds the *caiX* upstream region in a concentration dependent manner.** EMSA with biotin labeled *caiX* DNA probe alone (lane 1) or with increasing concentrations of purified MBP-CdhR (lanes 2-4). A cold *caiX* probe was used to compete for binding of MBP-CdhR from the labeled probe (lane 5). A nonrelated *dhc* DNA probe was used to show specificity of MBP-CdhR binding to *caiX* (lane 6-8).

**Figure 5. The CdhR binding site sequence and key residues for induction are identified from DNaseI footprinting and Miller assays.** (A) DNaseI footprinting assay was performed by taking the *caiX* promoter end labeled with P32 and adding increasing concentrations of MBP-CdhR. The samples were subjected to DNaseI treatment. The samples were run on a 5% polyacrylamide Tris-borate-EDTA gel and analyzed to determine the CdhR binding site. The first lane of the gel is the A+G sequencing ladder and the others are with or without MBP-CdhR. (B) The *caiX* enhancer site was mutated by changing two bases at a time in the *caiX* distal binding site to adenosines and fused to *lacZ* (pos, mut1-mut6). *P. aeruginosa* PA14 wild type carrying each of the plasmids were grown in MOPS with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$ , and induced with 1 mM carnitine for

4 hours and then subjected to Miller assays. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments. \*\*\*\*,  $P < 0.0001$

**Figure 6. MBP-CdhR binds but does not regulate *cbcX* expression.** (A) EMSA with a biotin labeled *cbcX* upstream region and purified MBP-CdhR in increasing concentration. (B) Relative expression of *cbcX* was calculated based on the expression in WT pyruvate normalized to the *rplU* transcript. Three biological samples were run in triplicate and the graph represents the mean values and SEM. n.s., not significant; \*\*\*,  $P < 0.001$ .

**Figure 7. MBP-GbdR binds the *caiX-cdhR* intergenic region but does not induce transcription of *caiX* or *cdhR*.** (A) EMSAs were performed with purified MBP-GbdR in increasing concentration with either the biotin labeled *cdhR* binding site probe or a *cdhR* mutant binding site probe. The mutated probe has the distal half site CG residues in relation to *cdhR* changed to AA. (B)  $\beta$ -galactosidase assay with a *caiX-lacZ* reporter plasmid (pJAM22) in wild type,  $\Delta cdhR$ , or  $\Delta gbdR$  strains were grown in MOPS, 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and induced cultures have an additional 1 mM carnitine. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments.

**Figure 8. CdhR promotes *cdhR* expression and GbdR dampens basal expression.** (A) PA14WT, PA14 $\Delta cdhR$ , PA14 $\Delta gbdR$ , and PA14 $\Delta cdhR\Delta gbdR$  carrying a *cdhR-lacZ*

translational plasmid reporter (pJAM135) were grown in MOPS with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and induced with 1 mM carnitine. The cultures were subjected to Miller assays. (B) PAO1WT, PAO1 $\Delta cdhR$ , and PAO1 $\Delta gbdR$ , all with the translational fusion *cdhRyfp* integrated at the *attTn7* site, were grown on MOPS agar pads with 20 mM pyruvate and with or without 1 mM carnitine. Cells were imaged under phase contrast and YFP fluorescence every 10 minutes for 6 hours at 32 °C. Cells were analyzed for mean fluorescence at each time point by subtracting the background fluorescence intensity for the cell fluorescence intensity.

A.



B.

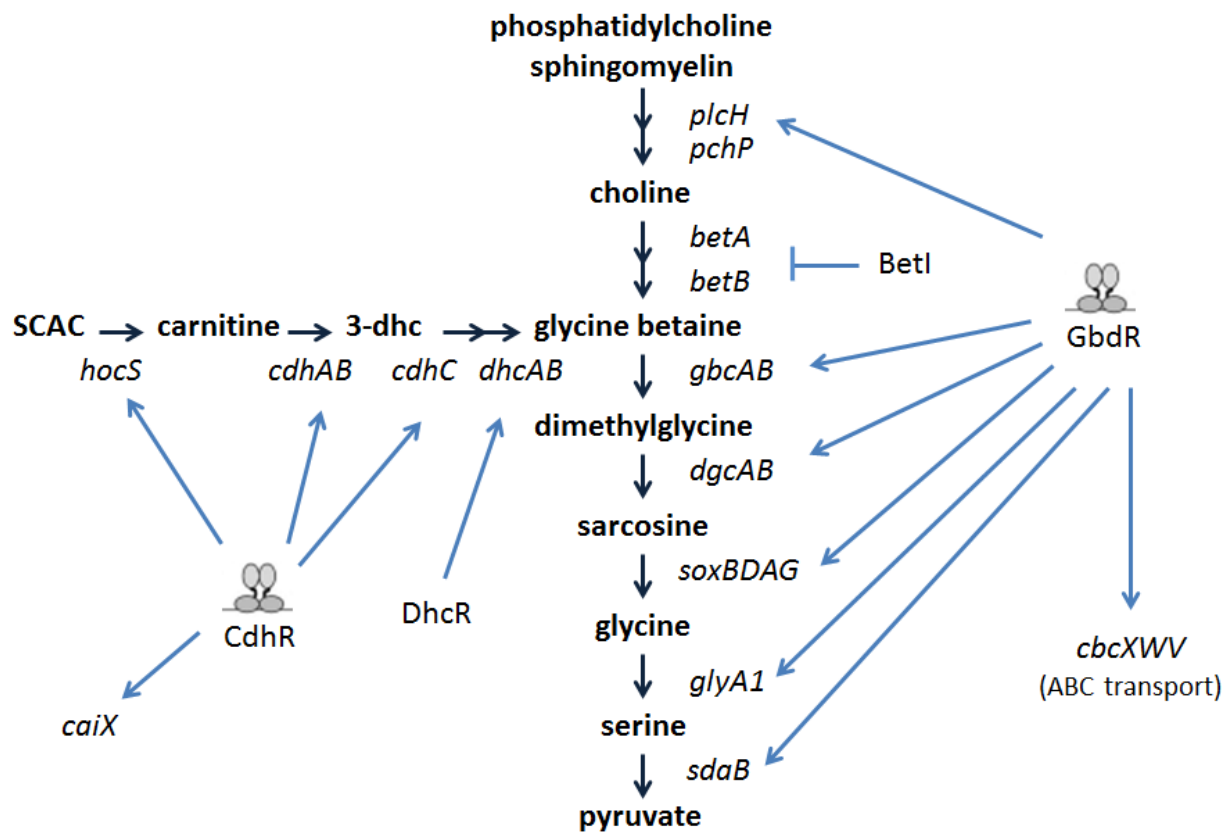
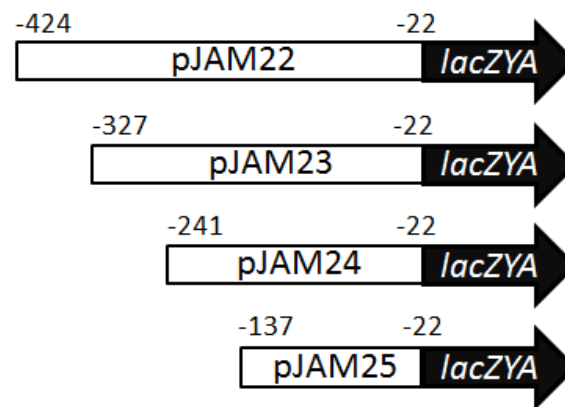


Figure 1. . Diagram of the *P. aeruginosa* PAO1 carnitine catabolism operon and the catabolic pathway.



A.



B.

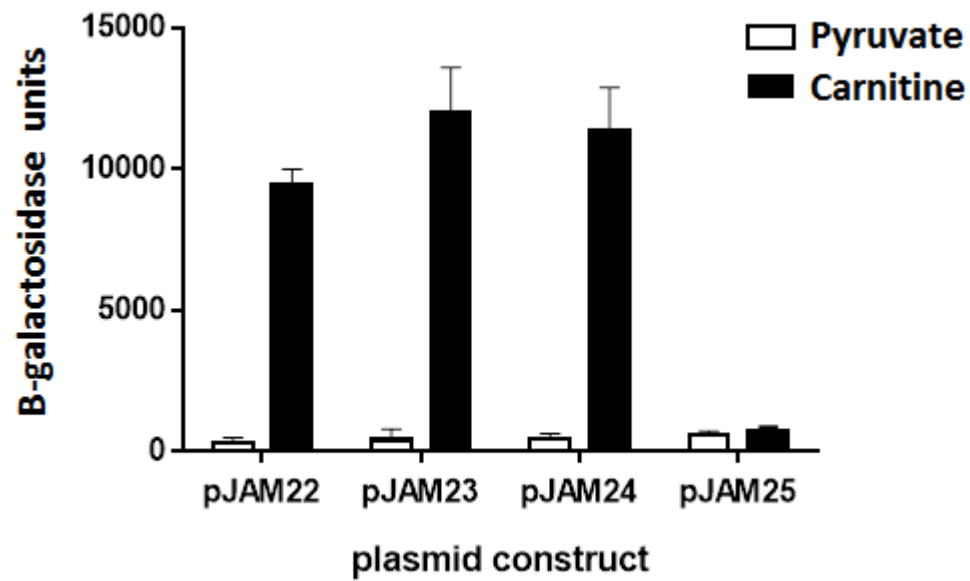


Figure 2. Promoter mapping of the upstream region of *caiX* exposes the CdhR binding site.

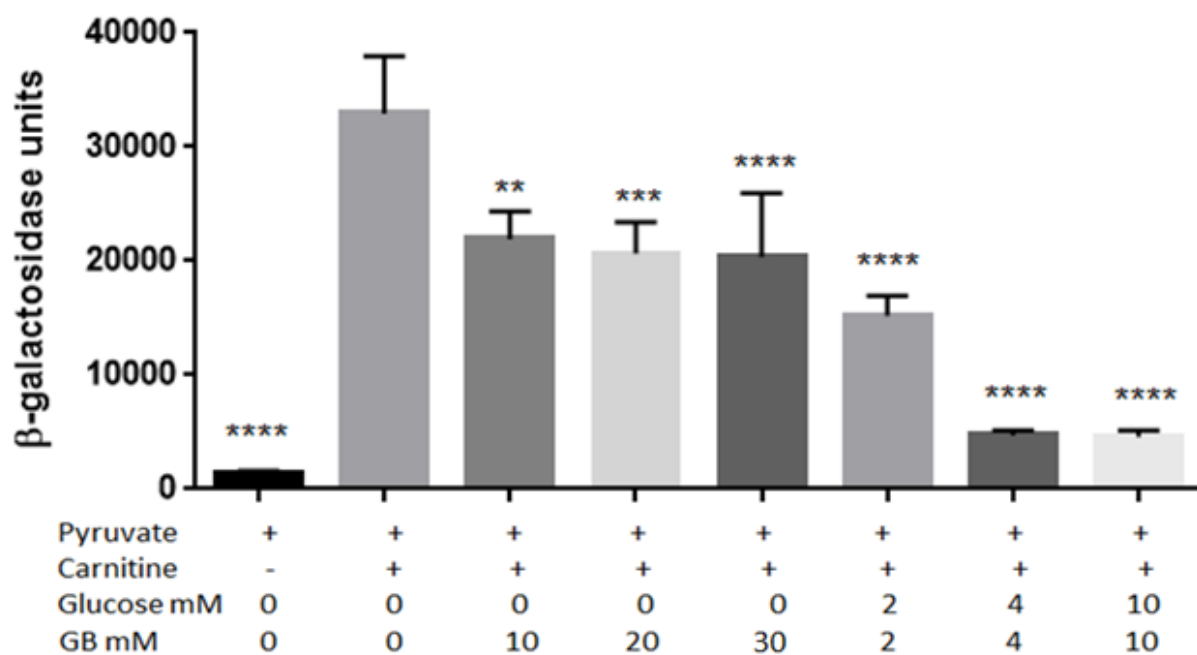
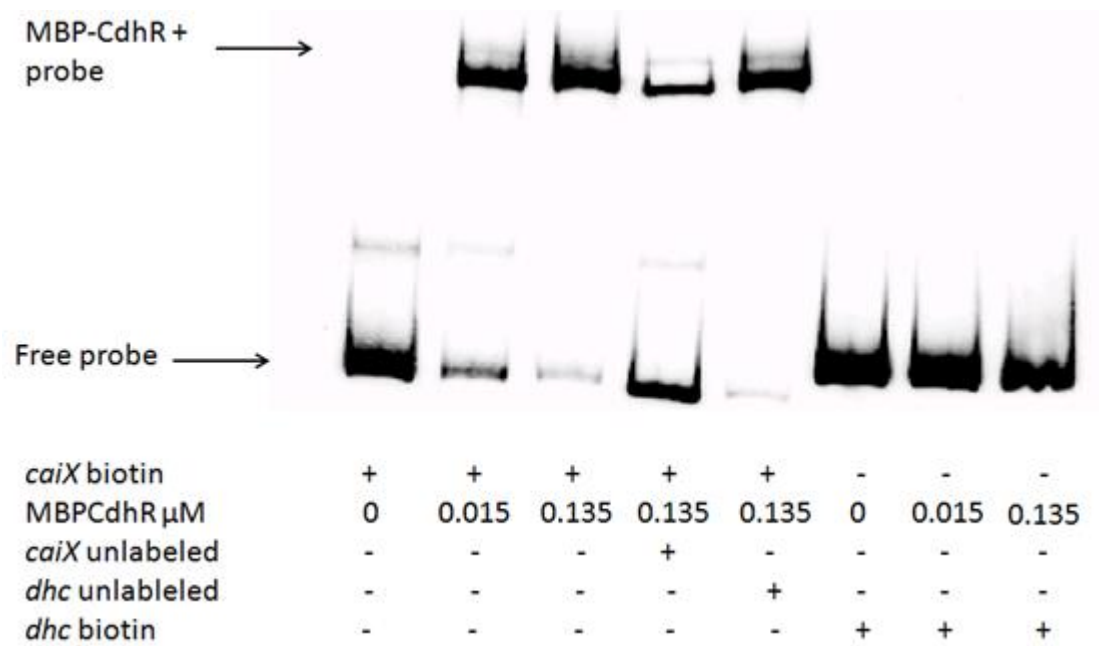


Figure 3. *caiX* transcription is repressed by glucose and glycine betaine.



**Figure 4. MBP-CdhR binds the *caiX* upstream region in a concentration dependent manner.**

A.



B.

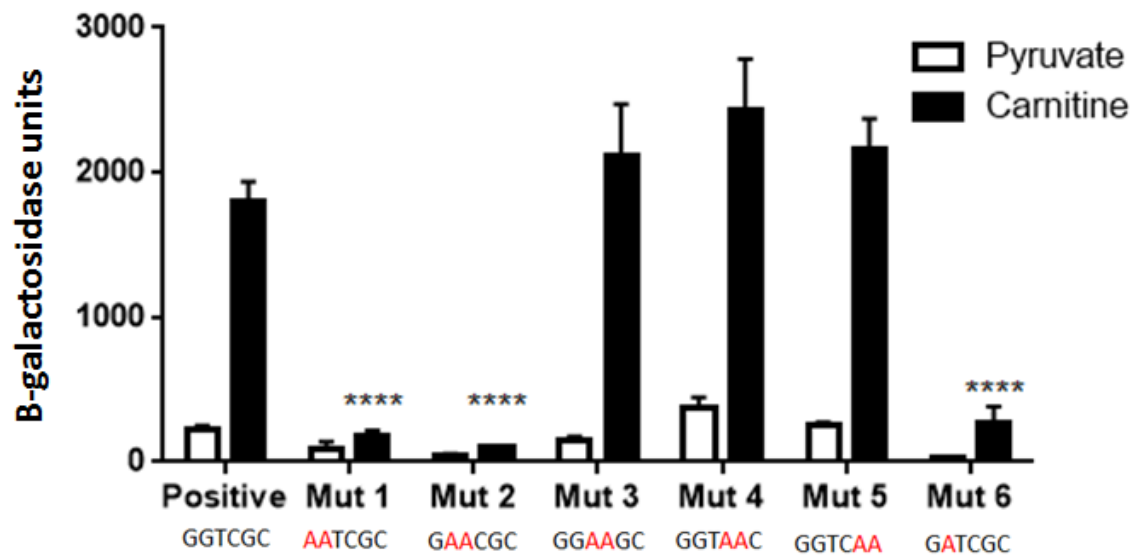
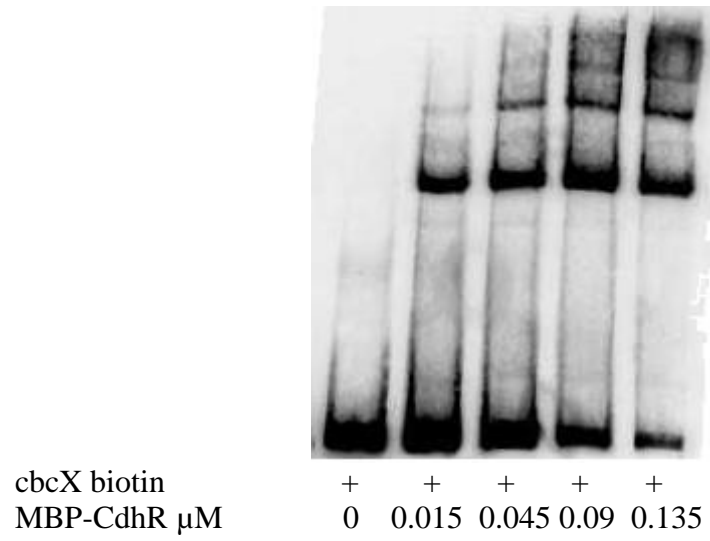
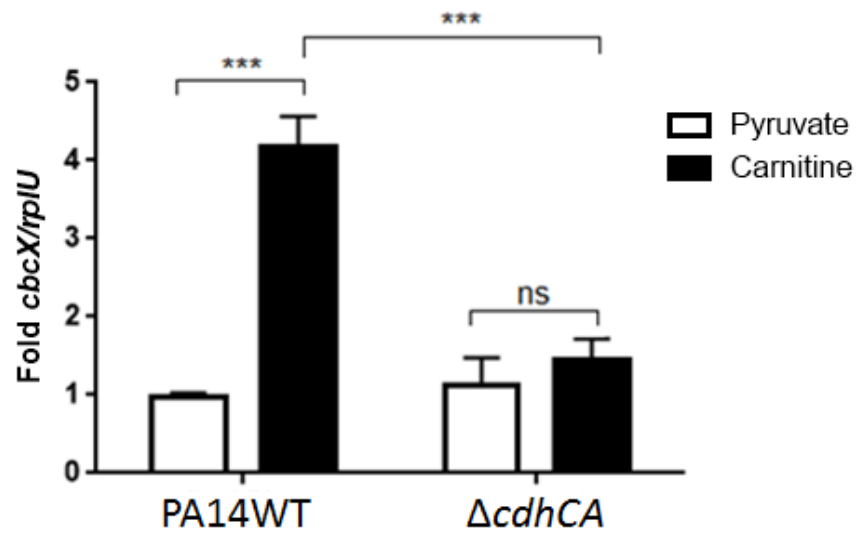


Figure 5. The CdhR binding site sequence and key residues for induction are identified from DNaseI footprinting and Miller assays.

A.

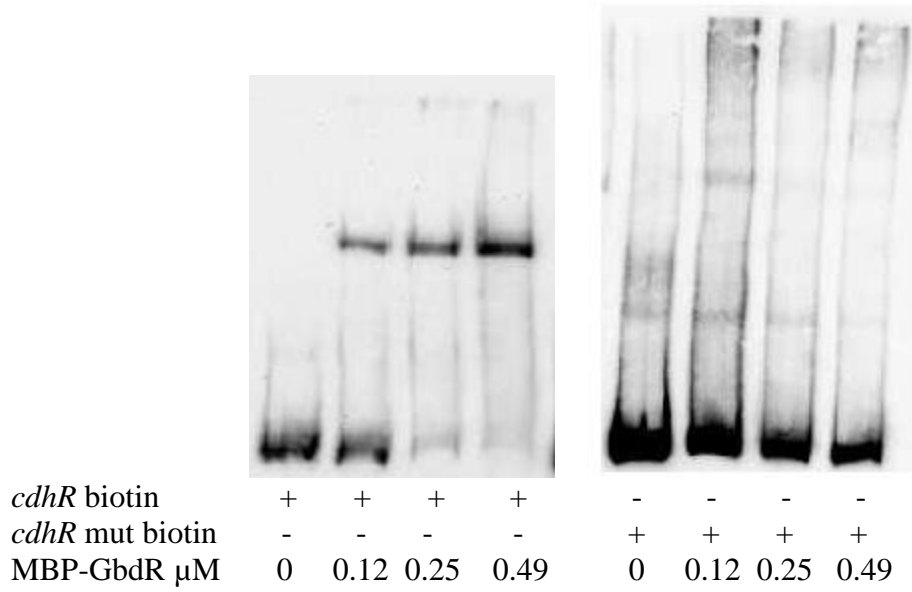


B.



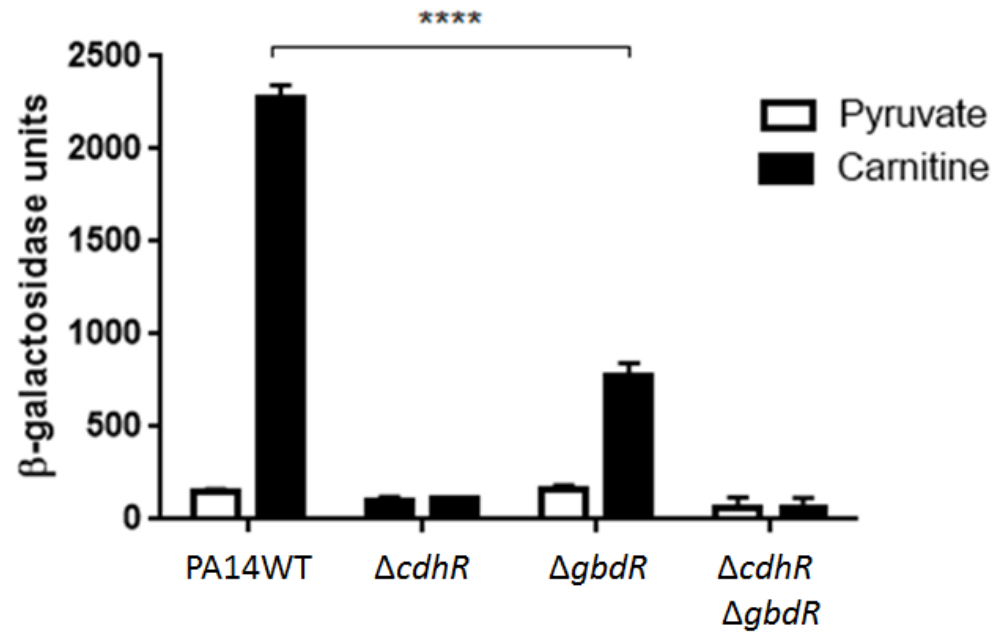
**Figure 6. MBP-CdhR binds but does not regulate *cbcX* expression.**

A.



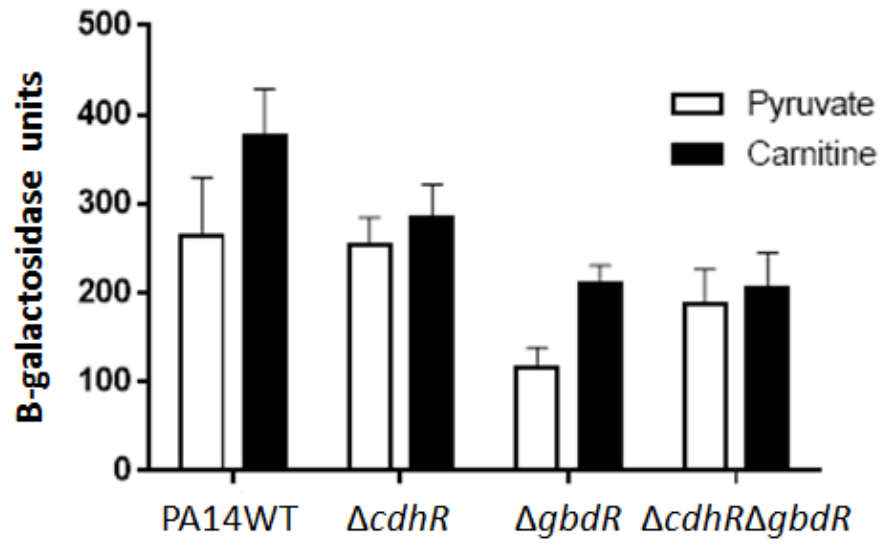
*caiX*---- ACGCCGCTGGTCGAGAACGTGACGTCGCTG ---- *cdhR*  
 \*\*

B.



**Figure 7. MBP-GbdR binds the *caiX*-*cdhR* intergenic region but does not induce transcription of *caiX* or *cdhR*.**

A.



B.

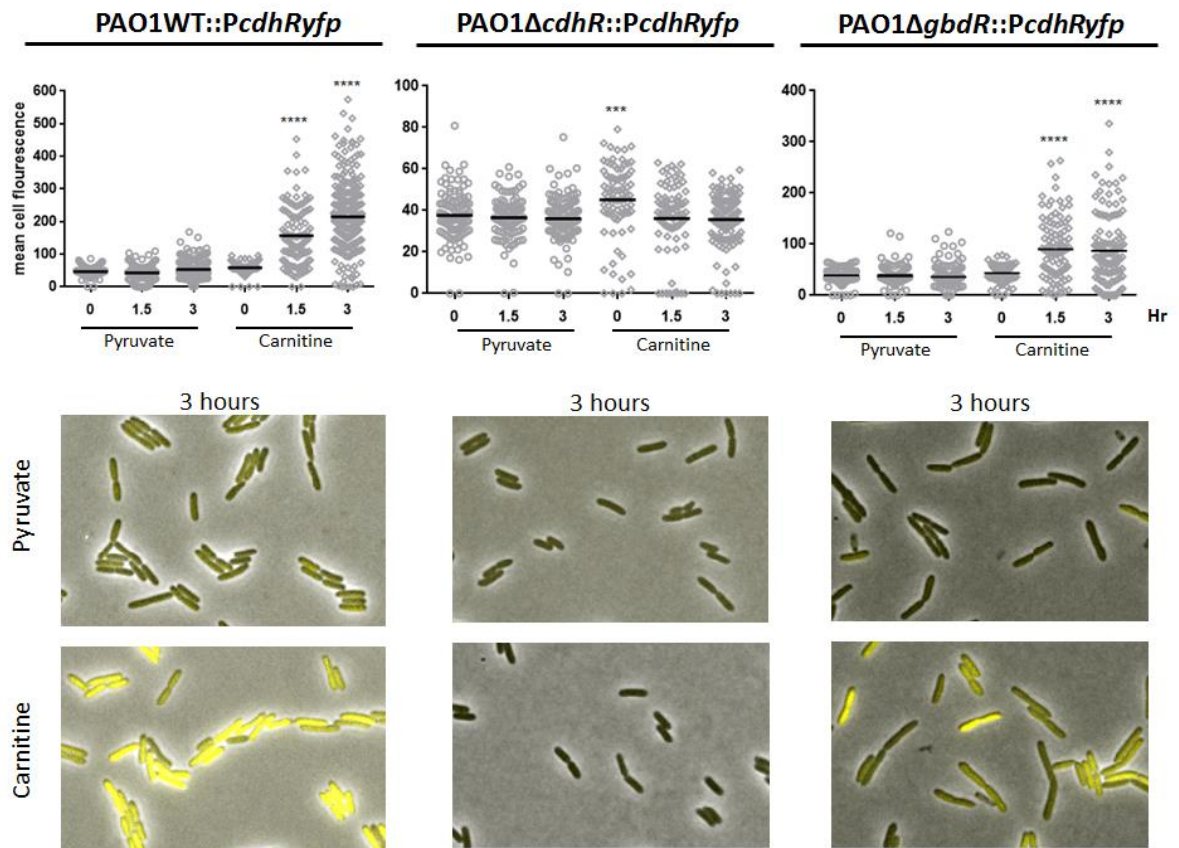


Figure 8. CdhR promotes *cdhR* expression and GbdR dampens basal expression.

#### 4.8. References

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## **CHAPTER 5:**

### **SUMMARY AND FUTURE DIRECTIONS**

## 5.1 Summary

This dissertation focuses on *Pseudomonas aeruginosa* sensing and utilization of the environmental and host-derived compound carnitine, as well as its acyl derivatives, acylcarnitines. Carnitine and acylcarnitines are widely distributed in nature and are especially abundant in animal tissues [1]. *P. aeruginosa* can catabolize carnitine [2] and we showed for first time that it is capable hydrolyzing acylcarnitines to use the liberated carnitine, and its catabolic product glycine betaine, as osmoprotectants, inducers of a virulence factor, and as sole carbon, nitrogen, and energy sources. We identified a short-chain acylcarnitine hydrolase, HocS, and determined how acylcarnitines are transported (Figure 1). Lastly, we expanded our understanding of how carnitine catabolism is regulated by the transcription factors CdhR and GbdR.

Figure 1 is a model representing this dissertation in its entirety. Although represented in the periplasm, medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically by an unknown hydrolase to yield free carnitine and a medium- or long-chain fatty acid. The fatty acid can be imported and used as a sole carbon source. The liberated carnitine and free carnitine, along with short-chain acylcarnitines are imported through the ABC transporter CaiXCbcWV for growth. Alternatively, carnitine can function as an osmoprotectant, whether from medium- and long-chain acylcarnitines or free, by being imported through an unknown transporter, likely a BCCT. Once in the cytoplasm, short-chain acylcarnitines are hydrolyzed by HocS. Finally, carnitine catabolism is controlled by the AraC family transcription factor, CdhR.

*Pseudomonas aeruginosa* is found in soil, water, and can thrive on surfaces such as medical devices. It adapts to different environments by utilizing a wide range of compounds for nutrients and survival, such as carnitine and its acyl derivatives. For the most part, *P. aeruginosa* is thought to be a free-living noninfectious microbe, but in the right environment can cause disease, typically in people who are immunocompromised. What is often overlooked is *P. aeruginosa*'s ability to cause infection in plants, insects, reptiles, and other mammals besides humans. Many pathogens cause disease in a specific host, whereas *P. aeruginosa* is a widespread and resourceful pathogen, likely owing to its large genome that encodes diverse metabolic pathways and a large regulatory repertoire [3]. *P. aeruginosa*'s ability to metabolize carnitine and acylcarnitines is not essential for survival, suggesting that metabolism of carnitine and acylcarnitine is an additional tool in the metabolism toolbox. The ability to adapt and change genetic profiles quickly provides *P. aeruginosa* an advantage for establishing and persisting in various niches, a trait that can drive disease.

We made large strides in understanding carnitine and acylcarnitine metabolism but there are still many questions remaining. The carnitine and acylcarnitine figure (Figure 1) has the majority of the transport and metabolism steps determined. Short-chain acylcarnitine transport and metabolism is complete, but the medium- and long-chain acylcarnitine hydrolase(s) still needs to be identified. The alternative carnitine transporter that confers osmoprotection, likely a BCCT, remains to be identified. And lastly, how carnitine is bound by the substrate binding protein, CaiX, and the carnitine regulator, CdhR, is still not understood.

## 5.2 Medium- and long-chain acylcarnitines (MCAC- LCAC)

### 5.2.1. Identifying the MCAC-LCAC hydrolase(s)

We were the first to show that *P. aeruginosa* is capable of using acylcarnitines as sole carbon and nitrogen sources [4]. *Alcaligenes* sp., *Pseudomonas putida*, and *Acinetobacter calcoaceticus*, to name a few, all have been shown to catabolize acylcarnitines of varying length but no responsible gene has been identified [5-7]. The short-chain acylcarnitine hydrolase, *hocS*, was the first gene of any acylcarnitine hydrolase gene to be ascertained [4]. We still have yet to determine the medium- and long-chain acylcarnitine hydrolase(s). There are three methods that could be used to identify the hydrolase(s). The first approach is to take an already existing *P. aeruginosa* PAO1 [8] or *P. aeruginosa* PA14 [9] transposon mutant library and screen separately for growth on a MCAC and a LCAC as sole nitrogen sources. The second approach is to perform a genetic screen by transposon mutagenizing *P. aeruginosa* PA14 wild type strain carrying the *PcaIX-lacZ* transcriptional reporter on the pJAM22 plasmid (strain JM92) and screen for loss of  $\beta$ -galactosidase activity in the presence of the MCAC or LCAC as a nitrogen source. The third would be microarray analysis on wild type *P. aeruginosa* grown on MCAC or LCAC as sole nitrogen sources to compare the expression profiles to the wild type strain grown with carnitine as the sole nitrogen source. Both MCAC and LCAC should be used in any of these screens, as there could be independent hydrolases for the two classes of acylcarnitines. *P. putida* can grow on 10C-16C fatty acid chain lengths [6] and *P. aeruginosa* may have long-chain acylcarnitine hydrolase similar to *P. putida* but acquired or maintained a MCAC hydrolase due to its

pathogenic nature. The three proposed screens have inherent and known positive controls: carnitine catabolism genes (*cdhCAB*), transport genes (*caiXcbcWV*), carnitine regulator (*cdhR*), and genes required for glycine betaine catabolism to pyruvate (Figure 1, Chapter 4). In the  $\beta$ -galactosidase assay, those transposon mutants that are in the listed genes will be white, in the growth assay there will be no growth, and in the microarray they will be induced.

### 5.2.2. Why not octanoylcarnitine?

I think a fascinating aspect and question of acylcarnitine catabolism is why *P. aeruginosa* doesn't use octanoylcarnitine as a sole carbon or nitrogen source [4]; after identifying the MCAC-LCAC hydrolase(s), this question can be addressed. The transport data in Chapter 3 shows that octanoylcarnitine is not hydrolyzed as efficiently as the other acylcarnitines. Although other bacteria can hydrolyze octanoylcarnitine [10], physiologic utility was generally not addressed except for *B. subtilis* employing the liberated carnitine as an osmoprotectant [11]. Interestingly, *P. aeruginosa* may have evolved a selective hydrolase as a survival strategy to combat the antimicrobial effects of caprylic acid [12-14], the direct product of octanoylcarnitine hydrolysis. Determining the protein structure or using gain-of-function mutagenesis to restore octanoylcarnitine would provide insight into the mechanism of octanoylcarnitine exclusion. Specific amino acid residues in the hydrolase may provide steric hindrance by interacting with the fatty acid and occluding octanoylcarnitine from the binding pocket.



### 5.3. Transport

#### 5.3.1. Transporter for osmoprotection

The observation that carnitine induces *caiX* and functions as an osmoprotectant in the absence of the ABC transporter *cbcWV*, suggests that there is one or more additional carnitine transporters. We hypothesize that carnitine can be imported through a betaine/choline/carnitine transporter (BCCT) particularly at high salt concentrations. The only characterized BCCT that is known to transport carnitine is *caiT* in *E. coli* and *P. mirabilis* [15, 16]. Since CaiT does not confer osmoprotection [17] and no homologs are predicted in *P. aeruginosa*, it is unlikely that *P. aeruginosa* uses a BCCT similar to CaiT. *P. aeruginosa* does possess BetT1, BetT2, and BetT3; BCCTs that are driven by proton motive force, transport glycine betaine and choline, and provide osmoprotection [18, 19]. These BetT transporters are good candidates for the alternative carnitine transporter. The *betT1*, *betT3*, *cbcVbetT1*, *cbcVbetT3*, and *betT1betT3* [18] mutants are available and would be a great starting point to fully understand carnitine transport. If the BetT proteins are not the alternative carnitine transporter, transposon mutagenesis of the *cbcV* mutant can be performed. Screening for lack of growth in a hyperosmotic environment will identify the carnitine specific BCCT.

#### 5.3.2. CaiX ligand specificity

The *P. aeruginosa* quaternary amine SBP CbcX binds choline and, to a lesser extent, glycine betaine [20]. Based on our growth data, CaiX is analogous to CbcX and binds multiple ligands: carnitine, acetylcarnitine, and butyrylcarnitine, and would be

interesting to see which of these ligands is preferred for CaiX. Using radiolabeled compounds and performing transport assays [20], would give insight into ligand preference. Carnitine is more prevalent than short-chain acylcarnitines in the environment [21] and animal tissues [22], which may be mirrored by the ligand preference of CaiX.

## **5.4. Regulation of carnitine catabolism**

### **5.4.1. Ligand binding**

It has been shown through reporter assays and via analysis of epistasis that CdhR is responsive to carnitine, but ligand binding has not been definitively shown. Ligand binding studies of AraC-family TF have been difficult due to the unruly nature of AraC TFs but binding has been shown in a few instances [21-23]. Using recombinant CdhR and radioactive carnitine, far western or equilibrium dialysis assays would show ligand binding by CdhR. It would be important to extend ligand binding studies to GbdR to tease apart the role GbdR has as a dominant regulator of carnitine and choline catabolism.

### **5.4.2. Catabolite repression of carnitine catabolism**

The role of GbdR in catabolite repression of carnitine catabolism is an intriguing question. Glucose and glycine betaine can both repress the transcription of *caiX*. For GB, this catabolite repression control is predicted to be important for negative feedback as carnitine is converted to GB. This repression may be regulated by GdbR, supported by our previous published data that GbdR responds to GB [26-28] and data presented here

showing that GbdR binds the *caiX-cdhR* intergenic region. Reporter experiments in a *gbdR* mutant will help elucidate the source of transcriptional repression, but will need to take into account GbdR-dependent activation of the carnitine and GB transporter ABC transporter *cbcWV* [20]. To circumvent the defect of GB and carnitine transport in a *gbdR* mutant, placing a constitutive promoter at the native locus of *caiXcbcXWV*, or having the GB-carnitine transport system *in trans* would alleviate a GB and carnitine transport defect.

#### **5.4.3. How is *cdhR* transcription regulated?**

There are a few things we know about the regulation of *cdhR* transcription: GbdR is not an activator of *cdhR* transcription and CdhR plays a role in its own regulation. A *cdhR* mutant with stop codons truncating the ORF maintains the transcript but not the protein, which allows us to assess transcript levels by qRT-PCR in a *cdhR* mutant. In Chapter 4, I propose a mechanism for CdhR and GbdR influencing transcription by regulating CdhR binding to the *caiX-cdhR* DNA region, either by binding half sites and looping the DNA or by protein oligomerization (Figure 2) along the DNA. There are additional CdhR and GbdR half sites in the intergenic region and within *caiX* and *cdhR* (Figure 3). I hypothesize that they contribute to the observed transcriptional repression. By strategically adding a reporter in the CdhR half site rich area and/or mutating certain half sites we can elucidate if these half sites function as operator sites. This can be expanded to GbdR but may prove to be difficult or very time

consuming because in Figure 3 I have marked the most common distal half site GbdR binding sequence, not the other possible GbdR binding sites.

## 5.5 Concluding remarks

Carnitine metabolism in *P. aeruginosa* has been known for over fifty years but it was not until recently that the genes responsible for carnitine catabolism were discovered; and it was not until this work that acylcarnitine metabolism was characterized in *P. aeruginosa* (Figure 1). We show how acylcarnitines are transported into the cell, and for the first time, identify the gene responsible for encoding a short-chain acylcarnitine hydrolase. Although the medium- and long-chain acylcarnitine hydrolase(s) is still unknown, there are clear benefits for *P. aeruginosa* maintaining an acylcarnitine degradation pathway, including the products being as osmoprotectants, to induce virulence factors, and to function as carbon, nitrogen, and energy sources. We further expanded upon the regulation of this system to include characterization of the CdhR binding site, investigation of *cdhR* expression, and exploration of the regulatory hierarchy between the transcription factors CdhR and GbdR.

Utilization of carnitine and acylcarnitines by *P. aeruginosa* contributes to its metabolic versatility and likely fine-tunes adaptation to various environments. Carnitine and acylcarnitines in soil and plants provide nutrients and osmoadaptation capabilities to survive the ever-changing environment, but may also provide *P. aeruginosa* an advantage in the human host and establishment of infection. Long-chain fatty acids are a carbon and energy source in mouse chronic wound infections [29], and these fatty acids

could partially be coming from long-chain acylcarnitine catabolism. Additionally, the carnitine catabolism genes are increased in expression by 5 to 15 fold compared to the succinate control in chronic wound infections [29]. These findings from Turner *et al.* lead to the hypothesis that carnitine and acylcarnitine metabolism are important for establishing infections where there is significant damage to areas high in carnitine, like muscle [1].

## 5.6. Figures

**Figure 1. Diagram of carnitine and acylcarnitine metabolism.** Abbreviations: OM is outer membrane, IM is inner membrane, SCAC is short-chain acylcarnitine, MCAC is medium-chain acylcarnitine, LCAC is long-chain acylcarnitine, HocML is hydrolase of *O*-acylcarnitine medium long, HocS is hydrolase of *O*-acylcarnitine short chain, FA is fatty acid, and BCCT is betaine/choline/carnitine transporter.

**Figure 2. Proposed mechanism of *cdhR* expression.** Red and purple bars are CdhR and GbdR half sites, respectively. Red monomers are CdhR and purple monomers are GbdR. Black diamonds are carnitine and yellow triangles are glycine betaine (GB). Blue and green lines are representations of *cdhR* and *caiX* expression levels, respectively.

**Figure 3. DNA sequence of *caiX* and *cdhR* with CdhR and GbdR half sites.** The DNA sequence colored green is the *caiX* sequence, red is the intergenic region, and blue is the *cdhR* sequence. The underlined and yellow highlighted sequence is the identified CdhR binding site. The GCGACC is the CdhR half site binding sequence and is underlined in black. CGCTG the GbdR distal half site and is in bold orange [26].

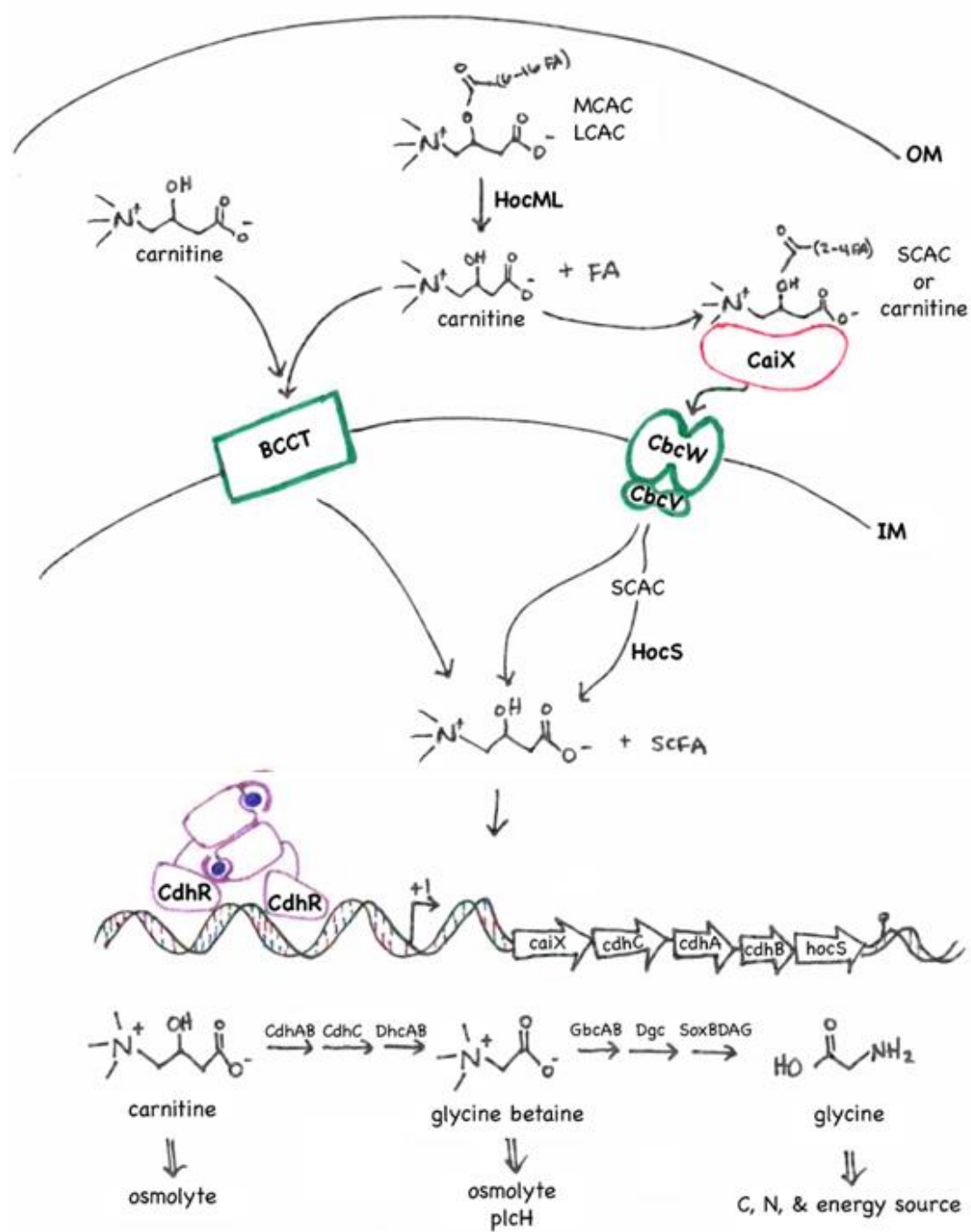
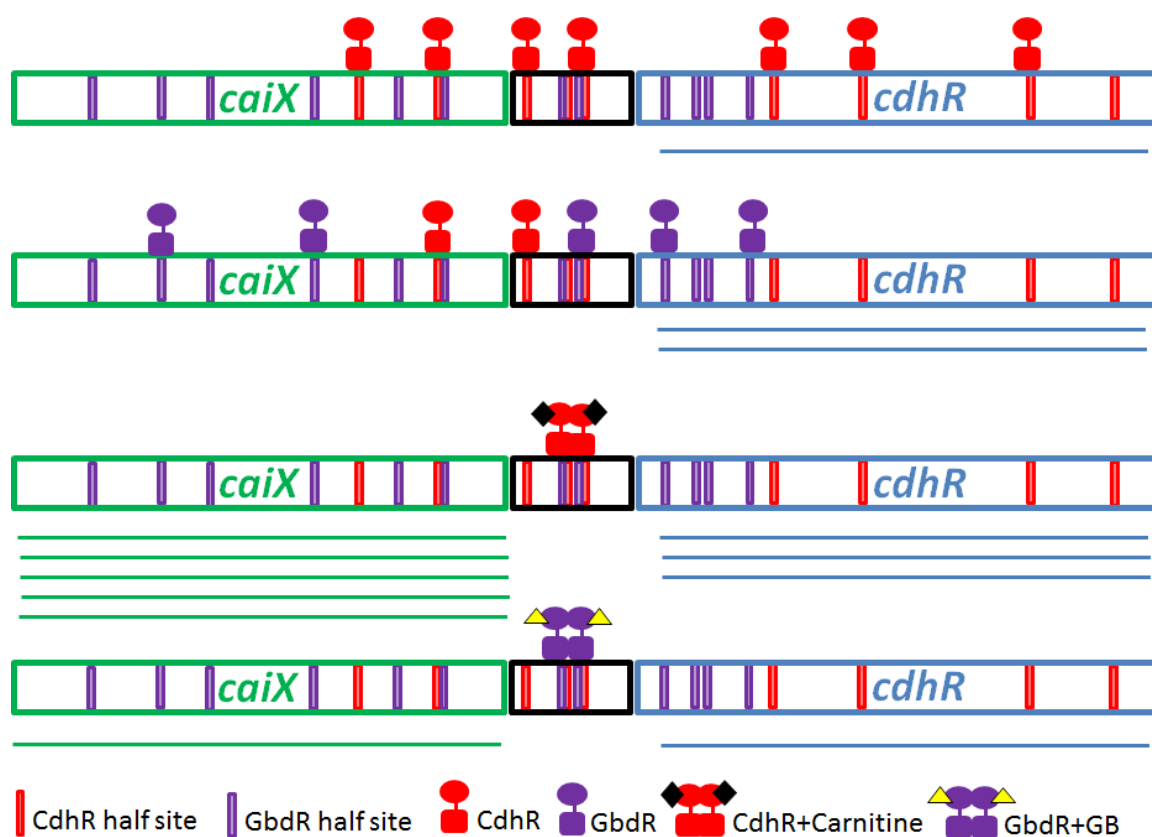


Figure 1. Diagram of carnitine and acylcarnitine metabolism.



**Figure 2. Proposed mechanism of *cdhR* expression.**



TTA<sup>CTT</sup>CAGCGAGG<sup>CCT</sup>TGGAGCGCGGCCAGGCCGT<sup>CCT</sup>TGCCGTCTGAAGGTGCTCACGCCATC  
 CAGCCAGGC<sup>CTT</sup>CAGCGGCTCGGGGTTGGC<sup>CTT</sup>CAGCCACTGGCGCGCGGCGTCGAGGGGCT  
 GGGTGC<sup>GAT</sup>CGAGGATCGGCGCCATCACCTGGCTGACCTGGG<sup>CGCTG</sup>CTGAAGCGCAGGTTG  
 TGCAGCAGCCGGGCGACGTTGCCGCACTGCGCCTGGTAGCCGGCGGCGGTCATGGTGGAGAC  
 GGTGGCCGCGCCCTCGTCGGGACCGAACACATCCT<sup>CGCTG</sup>CCGGTGAGGTAGGTCATGTCGA  
 TCTGCAGGTT<sup>CAT</sup>CGGATGCGGCTTCCAGCCGAAGAACACCACCCACTGCTTGC<sup>GCT</sup>TGATCG  
 CCCGCTTCACCGCCGTGAGCATCCC<sup>GGCCT</sup><sup>CGCTG</sup>GACTCCACCAGTTGGAAGCCCTTCAGGC  
 CGAAACGGTTGTCGTCGATCATCTTCCGGGTGATGCGGTTTCGAGC<sup>CGCTG</sup>CCCGGCTCGATGG  
 CGTAGATGCGGCCGCCAAGCTTGTCTTGAAGCGGGCGATGTCGGCGAAGGTCTTCAGCCCGC  
 CCTCGGCGACGTAGCTCGGCACCGCATAGGTGGACTGCGCGTCGGCCAGGGTCGGTGGCTCG  
 ATCACTTCGATCCGGCCTTTGTCCAGGTAAGGCCTG<sup>GCGACC</sup>GGCTGCATGGTCGGTTGCCAG  
 TAGCCGAGGAACAGGTCCAGCTGCTTGTTCGGCCAGGCCGGCCAAAACGATCTGTTGCGAGG<sup>C</sup>  
<sup>GCTG</sup>GTCTGCTTACCTGGTAGCCCAGGCCATCGAGCAAGGCTTCGGCCACGG<sup>CGCTG</sup>CTG<sup>G</sup>  
<sup>CGACC</sup>ACGTCGGTCCAGTTCACCGTGCCAGGCGCACCGTACGGCACTGCTCGGGTTCTGCGG  
 CCAGGGCGGCGCCACTGGCCAGGATCAGCAGGGCGCAGCCAGCTGGCGAATCGTCGTCTTC  
 ATCGGTCTCCCCTCGTGCGGCTATTGATTGTTTTCTGCGAGGTGGGCGCCGGCCCTTGCAG  
 ACCGACCCTG<sup>GCGACC</sup>AAGGTACGCAGCCGGGACGGCGGCGAAGCGCACTGCG<sup>GCGACCAG</sup>  
<sup>CTCTGCACTGCA</sup><sup>GCGACC</sup>TGGCCATTGCCAAGCGCGGCGGGCCTGCGCTACACACAGGCTG  
 CGCAACCTCAACCCCGGAGCGCCCCATGTCCCAGGACTTCTGGTTTCTCCTGCTGCCCGGCTTC  
 TCGGTAATGGGCTTCGTTTCCGCCGTGGAGCCACTGCGCGTGGCCAACCGCTTCCACGCCGAC  
 CTCTAC<sup>CGCTG</sup>GCACGTGCTCAGCGCCGACGGCGGTCCGGTGCTGGCCAGCAATGGCATGTC  
 GGTGAACAGCGACGGCG<sup>CGCTG</sup>GAGC<sup>CGCTG</sup>AAGAAAGGAGACCTGCTGTTCTGTGGTGGCC  
 GGTTTCGAGC<sup>CGCTG</sup>CGGGCGGTACCCCGGCACTGGTGCAGTGGCTGCGCAAGCTCGATCG  
 CAACGGCGTGACCCTCGGTGGCATCGATACCGGCAGCGTGGTGCTGGCCGAGGCCGGTCTGCT  
 CGACGGGCGCCGC<sup>GCGACC</sup>CTGCACTGGGAAGCCATCGACGCCTTCCAGGAGTCCTACCCGC  
 AACTGAGCGTGACCCAGGAGCTGTTTCGAGATCGACGGTCCGCGCATCACCTCGGCCGGCGGC  
 ACCGCCTCCATCGACCTGATGCTCGACCTGATCGCCAGGCCACGGTCCGCAACTGGCGGTG  
 CAGGTTTCCGAGCAGTTCGTGCTCGGCCGCATCCGCCGCGCCAGGACCACCAGCGCCTGCAG  
 GTG<sup>GCGACC</sup>CGCTATGGCGTGAGCAACCGCAAGCTGGTGCAGGTGATCGGCGAGATGGAGCG  
 GCATACCGAGCCGCCACTGACCACCCTGGAGCTGGCCGAGCGCATCCAGGTCACCCGCCGCC  
 AGCTGGAACGGCTGTTCCGCGTGACCTGGACGACACGCCCTCGAACTTCTACCTCGGCCTGC  
 GCCTGGACAAGGCCCGCCAGTTGCTGCGCCAGACCGACCTCAGCGTGCTACAGGTGAGCCTG  
 GCCTGCGGCTTCGAG<sup>GCGACC</sup>CTTCGTA<sup>CTT</sup>TCTCCCGCAGCTACCGCGCGCGCTTCGCCGCCAG  
 TCCCAGCCAGGACCGCGCGGTGCTGCCGCTCAAGGCTCCCGCAGCCACGCCGCCAGGCGCGC  
 CAGCAGGGC<sup>GCGACC</sup>ACGCCTCGAGCTGAGCGAGGCTGA

**Figure 3. DNA sequence of *caiX* and *cdhR* with CdhR and GbdR half sites.**

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## **CHAPTER 6:**

## **APPENDIX**

## 6.1. Rationale

The data presented below is pertinent to this thesis but is not incorporated into Chapters 2-4. Therefore, this section has some experiments briefly described and the results are stated.

## 6.2. Induction of *caiX* with varying concentrations of carnitine

To determine the sensitivity of *caiX* induction by carnitine, the concentration of carnitine was titrated and a Miller assay was performed. We were able to assess the inducing limit of *caiX* by carnitine.

*Pseudomonas aeruginosa* PA14 wild type carrying *PcaiX-lacZYA* (pJAM22) was grown overnight at 37 °C in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and 20 µg ml<sup>-1</sup> gentamicin. Cells were collected by centrifugation, washed, and inoculated into MOPS minimal media with 20 mM pyruvate, 20 µg ml<sup>-1</sup> gentamicin, and with or without carnitine ranging from 1 mM to 250 nM. Cells were induced for 4 hours at 37 °C while shaking. A β-galactosidase assay was performed according to the method of Miller in duplicate.

The induction of *caiX* is similar from 1 mM to 250 µM carnitine. 125 µM of carnitine induces *caiX* to about 70 % of 250 µM and there is a sharp decrease in induction of *caiX* at 1 µM (Figure 1). Therefore any of the experiments performed in this thesis, whether it is 1 mM or 500 µM of the inducing agent, does not alter the relative induction observed. This is noted because there are some experiments have inducing compounds at 1 mM of the L-isomer compound compared to 1 mM DL-racemic mixture.

### 6.3. CdhR senses and responds to acetyl- and butyrylcarnitine

Short-chain acylcarnitines, acetyl- and butyrylcarnitine, are imported into the cell via the ABC transporter CaiXcbcWV as described in Chapter 4. Once in the cytoplasm the carnitine catabolism operon, *caiXcdhCABhocS*, is induced. The question addressed was does CdhR sense short-chain acylcarnitines and induce *caiX*, or do they need to be hydrolyzed first.

*Pseudomonas aeruginosa* PA14 wild type and  $\Delta hocS$  carrying *PcaiX-lacZYA* (pJAM22) were grown overnight at 37 °C in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and 20  $\mu\text{g ml}^{-1}$  gentamicin. Cells were collected by centrifugation, washed, and inoculated into MOPS minimal media with 20 mM pyruvate, 20  $\mu\text{g ml}^{-1}$  gentamicin, and induced with 1 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cells were induced for 4 hours at 37 °C while shaking. A  $\beta$ -galactosidase assay was performed according to the method of Miller in triplicate, three independent times.

Both acetyl- and butyrylcarnitine induce *caiX* in a *hocS* mutant similar to wild type (Figure 2). These data indicate that short-chain acylcarnitines are likely sensed by CdhR and do not need to be hydrolyzed to induce *caiX*.

### 6.4. Purification of CdhR and GbdR

CdhR and GbdR are members of the AraC family transcription factors (TF). As described in Chapter 1, AraC TFs have an N-terminal ligand binding and dimerization domain. In order to study ligand binding and protein interactions, it is beneficial to have

the protein in its native conformation and not have a protein fused to the N-termini. To purify the protein without any tags we perform affinity purification with heparin. Heparin is glycoaminoglycans that is negatively charged and structurally and charged similar to DNA. CdhR and GbdR are DNA binding proteins (the C-terminal domain is the DNA binding domain) and therefore heparin can be used to purify the TFs (Figure 3).

CdhR was constructed by PCR amplifying with primers 5'-TCAcataTGTCCCAGGACTTCTGGT and 5'-ACTggtacCGTCAGCCTCGCTCAGCTC using PA14 as template DNA. The product was digested with NdeI and KpnI and ligated into similarly cut pET30a. GbdR was constructed by amplifying with primers 5'-ACGcatATGACCACGTACGCGCCC and 5'-ACTggtaccTCAGATCCGCACGCTGGCG using PA14 as the template DNA. GbdR PCR product was digested with NdeI and BamI and ligated into the similarly cut pET30a. Both CdhR and GbdR carrying plasmids, pJAM91 and pJAM100, respectively, were transformed into T7Express *E. coli* and selected on LB 100  $\mu\text{g ml}^{-1}$  kanamycin plates. *E. coli* strain JM256 (CdhR) and JM273 (GbdR) were grown overnight in LB 100  $\mu\text{g ml}^{-1}$  kanamycin and inoculated into 1 L LB with 75  $\mu\text{g ml}^{-1}$  kanamycin. Cells were grown at 37 °C to an OD<sub>600</sub> nm of 0.4, induced with 1 mM IPTG, and induced for 3 hours. Cells were collected by centrifugation and resuspended in 3 ml 20 mM TrisHCl with 150 mM NaCl, Halt cocktail protease inhibitor (Thermo), and lysed by French press. Lysate was spun at 4 °C for 30 minutes and the supernatant was syringe filter sterilized with a 0.22  $\mu\text{m}$  filter and applied to a heparin column. The column was washed in wash buffer (100 mM PBS, 150 mM NaCl, pH 7.4) with an additional 350 mM NaCl and then eluted with wash buffer plus 500 mM NaCl. Elution fractions were



dialyzed in 20 mM TrisHCl, 150 mM NaCl, 1 mM EDTA, at pH 7.4 overnight in a 20K MWCO slidealyzer (Thermo). A Bradford assay was performed to determine protein concentrations.

## 6.5. Figures

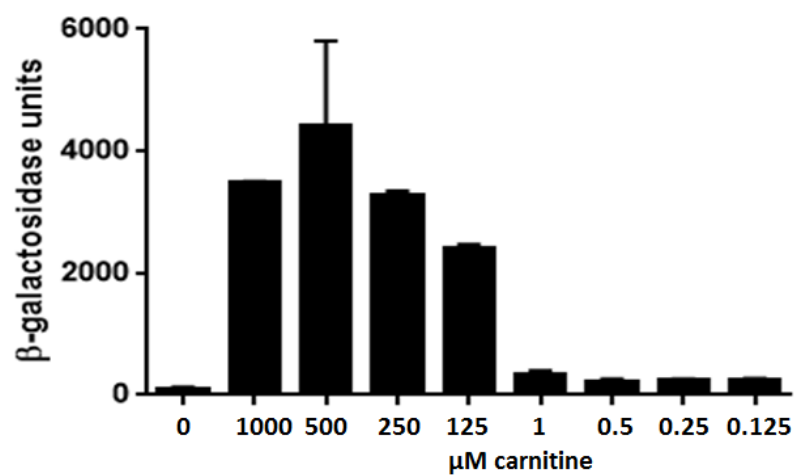
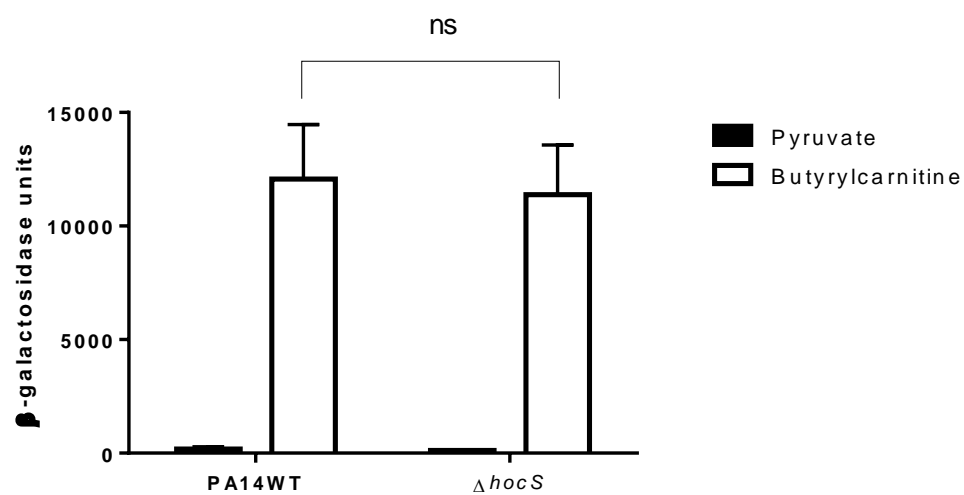
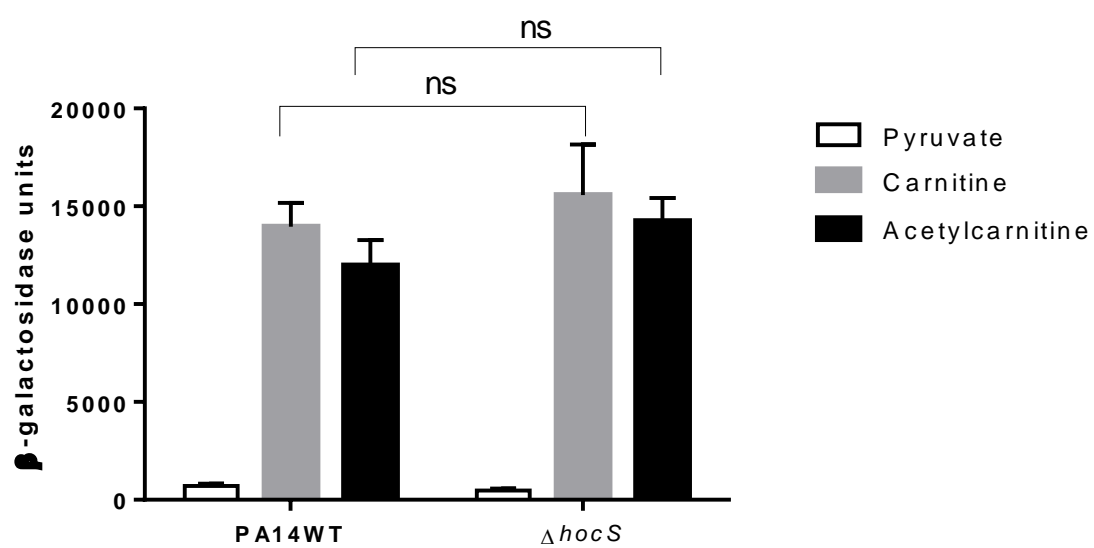
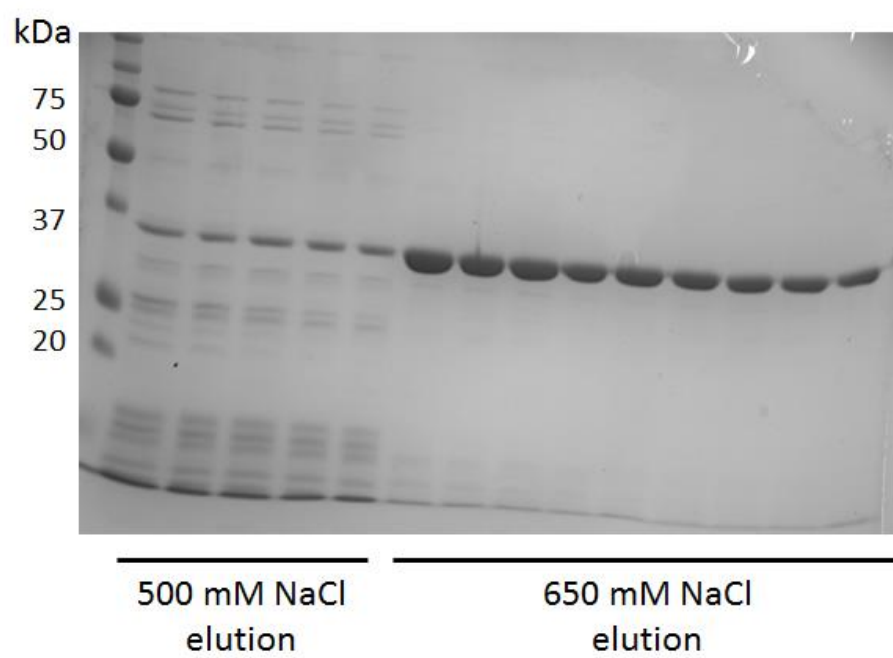


Figure 1. Titration of carnitine inducing *caiX*



**Figure 2. Acetyl- and butyrylcarnitine induce *caiX* in a *hocS* mutant**



**Figure 3. SDS-PAGE of purified CdhR**

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